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PATENT
029381.49884US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT(S): KRISTIINA YLIHONKO ET AL.

SERIAL NO.: 09/830,994 GROUP: 1652

FILED: May 3, 2001 EXAMINER: Kathleen M. KERR

FOR: THE GENE CLUSTER INVOLVED IN ACLACINOMYCIN BIOSYNTHESIS, AND ITS USE FOR GENETIC ENGINEERING

DECLARATION SUBMITTED UNDER 37 C.F.R.§1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, M.Sc. Kaj Räty, do hereby declare the following. I am one of the inventors of the subject matter disclosed in the above-captioned application.

I have reviewed the Office Action of February 11, 2004 and the Examiner's interpretation of the definition of sequence homologies.

The homology percentage (84%) as defined in Claim 2 is based on sequence analyses carried out when preparing the above-captioned patent application. First, we determined an average identity of the whole gene cluster in amino acid level, and then multiplied it by factor 1.333 to take account of the third wobbly base. The rationality of this manner is demonstrated in the enclosed Table showing identity percentages of the sequences. While we claim for 84% sequence homology, the best identity found in individual genes is 71%. Consequently, all of these identities are less than that given in Claim 2. The primary comparisons are made for amino acid sequences, since nucleotide sequences are always too dissimilar.

Consequently, according to my understanding claiming for 84% homology is in line with the assumption that if higher homology will be found the gene is involved in biosynthesis of aclacinomycins.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Kaj Räty

June 7, 2004
Date

Table: Homology search in 1999

Gene	Amino acids	Deduced function	Id %	Id% *1.333
sga1	662, not complete	unknown	50	67
sga2	272	activator	55	73
sga3	434	dehydratase	71	95
sga4	329	oxidoreductase	64	85
sga5	323	dTDP-glucose 4,6-dehydratase	67	89
sga6	443	glycosyl transferase	54	72
sga7	443	putative isomerase	39	52
sga8	267	aklaviketone reductase	65	87
sga9	144	putative polyketide assembler	51	68
sga10	259	cyclase	71	95
sga11	238	aminomethylase	55	73
sga12	291	glucose-1-phosphate thymidylyltransferase	68	91
sga13	341, not complete	aminotransferase	77	103

PATENTTI- JA REKISTERIHALLITUS
NATIONAL BOARD OF PATENTS AND REGISTRATION

Helsinki 7.8.2000

E T U O I K E U S T O D I S T U S
P R I O R I T Y D O C U M E N T

Hakija Applicant	**Galilaeus Oy** **Piispanristi**
Patenttihakemus nro Patent application no	**19992085**
Tekemispäivä Filing date	**29.09.1999**
Kansainvälinen luokka International class	**C12N**
Keksinnön nimitys Title of invention	

"The gene cluster involved in aclacinomysin biosynthesis, and its use for genetic engineering"
(Aklasinomysiinin biosynteesiin liittyvä geeniryhmittymä ja sen käyttö-geenitekniikassa)

Täten todistetaan, että oheiset asiakirjat ovat tarkkoja jäljennöksiä patentti- ja rekisterihallitukselle alkuaan annetuista selityksestä, patenttivaatimuksista, tiivistelmästä ja piirustuksista.

This is to certify that the annexed documents are true copies of the description, claims, abstract and drawings originally filed with the Finnish Patent Office.

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Claims

1. Isolated and purified DNA fragment, which is the gene cluster for the anthracycline biosynthetic pathway of the bacterium *Streptomyces galilaeus*, being included in a 7 kb *Xho*I–*Not*I fragment and a flanked 8.5 kb *Bgl*II fragment of *S. galilaeus* genome.

2. DNA fragment of claim 1, which comprises the nucleotide sequence given in SEQ ID NO:14, or a part thereof, or a sequence showing at least 84 % homology to said sequence.

3. A recombinant DNA, which comprises the DNA fragment of claim 1 or 2, or a part thereof, cloned in the plasmid replicating in *Streptomyces* or in *E. coli*.

4. The recombinant DNA of claim 3, which is the plasmid pSgs4 deposited in *S. lividans* strain TK24/pSgs4 with the accession number DSM 12998.

5. The recombinant DNA of claim 3, which is the plasmid pSgc5 deposited in *E. coli* strain XL1BlueMRF'/pSgc5 with the accession number DSM 12999.

6. Use of the genes derived from the DNA fragment of claim 1 or 2 in the production of anthracycline metabolites.

7. Use of the genes derived from the DNA fragment of claim 1 or 2 to increase aclacinomycin production.

8. Use of claim 6 or 7, wherein the genes are encoding an activator, a dehydratase, an oxidoreductase, a dTDP–glucose 4,6–dehydratase, a glycosyl transferase, an isomerase, an aklaviketone reductase, a polyketide assembler, a cyclase, an aminomethylase, a glucose–1–phosphate thymidylyl transferase, and an aminotransferase.

9. A process for increasing aclacinomycin production in a bacterial host, comprising transferring the DNA fragment of claim 1 or 2 into a *Streptomyces* host, cultivating the recombinant strain obtained, and isolating the aclacinomycins produced.

10. The process of claim 9, wherein the *Streptomyces* host is a *Streptomyces galilaeus* host.

11. The process of claim 10, wherein the *Streptomyces galilaeus* host is a mutant strain derived from *S. galilaeus* ATCC 31615.

12. A process for production of metabolites, comprising transferring the DNA fragment of claim 1 or 2 into a *Streptomyces* host, cultivating the recombinant strain obtained, and isolating the compounds produced.

13. A process for production of anthracycline metabolites, comprising transferring the DNA fragment according to claim 1 or 2 into a *Streptomyces peucetius* host, cultivating the recombinant strain obtained, and isolating the compounds produced.

A general approach for cloning and characterizing dNDP-glucose dehydratase genes from actinomycetes

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Key words: Actinomyces, Streptomyces, PCR, 6-Deoxyhexose, Genetic screening of dNDP-glucose dehydratases

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Abstract

Oligonucleotide primers were designed and successfully applied to amplify DNA fragments of dNDP-glucose dehydratase genes from actinomycete species producing natural compounds which contain deoxysugar moieties. The deduced amino acid sequence of the isolated fragments revealed similarity to known dNDP-glucose dehydratases. A phylogeny for the deduced proteins of the obtained fragments and for dNDP-glucose dehydratases described in the data bases was constructed. dNDP-glucose dehydratases from actinomycetes were more related to each other than to dehydratases from species of other orders. The phylogenetic analysis also revealed a close relation between dehydratases from strains producing natural compounds with similar deoxysugar moieties.

1. Introduction

Most of the occuring antibiotics consist of polypeptide, sugar or polyketide moieties. Due to the variety and combination of these moieties many antibiotics show interesting biological and pharmaceutical important activities. The majority of these compounds are produced by actinomycetes, many of them by the genera *Streptomyces* [1]. The incorporation of a deoxysugar as a structural component is modifying the surface properties of a compound and is influencing the way in which the compound interfaces with its surrounding [2]. In addition the precise function of the carbohydrate moiety has been elucidated in some cases [3, 4, 5, 6]. These examples include the interaction of oligosaccharide moieties to DNA to form stable complexes or the function of oligosaccharides as important immunological determinants. It has been demonstrated from studies on more than 100 pathways in *Streptomyces*, that antibiotic biosynthesis genes are clustered in one single chromosomal segment. Since different moieties are produced through different multi-step pathways, DNA fragments encoding biosynthetic genes range from 20-200 kb. In most organisms that produce antibiotics containing 6-deoxyhexose moieties, the genes that encode 4,6-dehydratases are found within the

biosynthetic gene clusters [7, 8, 9]. These dehydratase genes are highly conserved in actinomycetes. DNA probes derived from the genes encoding the streptomycin biosynthetic enzymes , StrD, E, L , M in *Streptomyces griseus* N2-3-11 (*S. griseus* N2-3-11), have been used to detect DNA fragments that presumably contain genes encoding enzymes responsible for the formation of 6-deoxysugars in some actinomycete strains [10]. In this paper we report a PCR method which can be used for the rapid amplification of DNA fragments from 4,6-dehydratase genes from a wide range of actinomycete strains. The experiments described in this report illustrate a reliable method for prescreening microorganisms for those producing compounds with deoxysugar moieties.

2. Material and methods

2.1 Bacteria strains and cultivation

The strains investigated in this study are listed in Table 1. The strains were grown as described in the references.

2.2 Polymerase chain reaction (PCR) conditions

PCR was performed on a GeneAmp 2400 thermocycler from Perkin Elmer, Weiterstadt, using Taq DNA polymerase and 10x reaction buffer containing $MgCl_2$ (2mM) from Perkin-Elmer. The starting denaturing temperature was 96°C for 4 minutes. The following 35 cycles lasted 3 minutes each. The denaturing temperature was 95°C (1.5 min) and the annealing-extension temperature was 72°C (1.5 min). At the end a final extension temperature was 72°C for 10 min. It was important to employ gelatine (1%) in the reaction mixture. The concentration of chromosomal DNA was 0.2 µg/100 µl incubation volume. Primers were used at a

concentration of 1 μM (equimolar) and deoxyribonucleoside 5′ triphosphates (dNTPs) were used at a final concentration of 200 μM. The primers were synthesized by Perkin-Elmer.

Sequence of primer 1:

5′ CSGGSGSSGCSGGSTTCATSGG 3′

Sequence of primer 2:

5′ GGGWRCTGGYRSGGSCCGTAGTTG 3′

(R: AG; W: AT; Y: CT; S: CG))

2.3 DNA manipulation

General procedures for manipulating DNA, such as preparation of streptomycete genomic DNA, restriction and transformation were carried out according to published methods [11, 12]. PCR fragments were isolated from an agarose gel using the Jetsorb kit from Genomed, Bad Oeynhausen or the Sure Clone Ligation kit from Pharmacia, Uppsala. Fragments were ligated into p-Bluescript SK⁻ (Stratagene, Heidelberg) or into pUC derivates supplied in the ligation kit. Sequence data were analyzed by using an automatic sequencer from Molecular-Dynamics, Krefeld, or Pharmacia, Uppsala. Colony hybridization was carried out using the non- radioactive hybridization kit from Boehringer, Mannheim.

2.4 Sequence comparison and phylogeny determination

The polypeptide sequences were compared and a phylogeny was constructed using DNASIS for windows, version 2, (Hitachi, San Bruno) scoring with a gap penalty of 5.0, a K-tuple of 2.0, a fixed gap penalty of 10.0 and a floating gap penalty of 10.0. The No. of top diagonals and window size were both set to 5.

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3. Results and Discussion

3.1 Design of PCR primers for the amplification of dTDP-glucose 4,6-dehydratases

The application of PCR has been proven a valuable tool for the identification of *Streptomyces* genes. Due to the biassed codon usage of Actinomycetes [13] oligonucleotide primers could be designed rather unambigiously even from short consensus sequences determined by amino acid comparison [14, 15]. The comparison of the sequence of known dTDP-glucose 4,6-dehydratases from *S. griseus* N2-3-11 [7], *S. violaceoruber* (Tü22) [9], and *Saccharopolyspora erythraea* (DSM5908) [16] revealed several regions of high similarity. One region is located at amino acid 6-15 of the proteins, a second one between amino acid 178 and 188 (Figure 1). Based on these sequences two oligonucleotide primers were synthesized taking into account the codon usage of actinomycetes. These primers were used to amplify DNA fragments from eight different actinomycete species producing a variety of different compounds (Table 1). The size of the amplified fragments was > 500 bp in each case which is a convenient size if the PCR fragment has to be labelled to be used in hybridization experiments.

3.2 Identification and characterization of dehydratase gene fragments

PCR fragments obtained from strains listed in Table 1 were subcloned and sequenced. Several subclones were investigated in each case. The sequences were analysed by codonpreference [17]. The deduced amino acid sequences of the isolated fragments revealed remarkable similarity to each other and to the dNDP-glucose dehydratase (StrE) isolated from *S. griseus* N-2-3-11. A multialignment of the derived amino acid sequences of the cloned dNDP-glucose-4,6-dehydratase gene fragments is given in Figure 2. The sensitivity of this method was demonstrated using *S. olivaceus* (Tü2353), the producer of elloramycin, a permethylated L-rhamnose containing polyketide antibiotic. Chromosomal DNA of *S. olivaceus* (Tü2353) did not hybridize to a dehydratase gene (*strE*) from *S. griseus* N2-3-11. [10]. However, we could

amplify and clone a PCR fragment which presumably is coding for a dNDP-glucose-4,6-dehydratase. Genes encoding enzymes involved in the synthesis of composed antibiotics are closely linked as demonstrated for *S. violaceoruber* (Tü22) which synthesizes the antibiotic granaticin consisting of a polyketide and a deoxysugar moiety [9]. Therefore the PCR fragments from *S. cyanogenus* S136 (DSM5087), *S. fradiae* (Tü2717), and *S. viridochromogenes* (Tü57) were used as probes in colony hybridization experiments. Cosmid DNA isolated from colonies hybridizing to the dehydratase probes were further analyzed by random sequencing. In addition to the dehydratase genes polyketide genes were identified in each case. The dehydratase genes were located approximately up to 10 kb from the genes encoding the polyketide synthetases indicating that the isolated dehydratase genes are involved in the biosynthesis of urdamycin A, landomycin A and avilamycin A, respectively (data not shown).

3.3 Evolutionary relationship of dehydratases

A phylogeny for the deduced proteins and for dNDP-glucose dehydratases described in the data bases was constructed. dNDP-glucose dehydratases from actinomycetes are closely related and differ from dNDP-glucose dehydratases from other bacteria (Figure 2).

The phylogenetic analysis also revealed an interesting relation between dehydratases from strains producing similar natural compounds:

(i) Among the different dehydratases the dehydratases isolated from S. cyanogenus S136 (DSM5087), *S. fradiae* (Tü2717) and *S. violaceoruber* (Tü22) are closely related to each other. These strains are producing natural compounds consisting of the deoxysugars D-olivose and L-rhodinose which are connected to a polyketide moiety.

(ii) It has been shown that the dehydratase genes isolated from the aminoglycoside producers *S. griseus* N-2-3-11 and *S. glaucescens* (DSM 40716) are involved in the biosynthesis of streptomycin and hydroxystreptomycin [7, 18]. The phylogenic tree analysis revealed a close

relation of both dehydratases to each other and a relation of both to a dehydratase from *S. ghanaensis* (ATCC14672). This strain is producing the phosphoglycopeptide antibiotic moenomycin A which, like streptomycin and hydroxystreptomycin, contains amino sugar moieties.

(iii) The dehydratases from *Saccharopolyspora erythraea* (NRRL2338) and *Amycolatopsis mediterranei* (DSM5908) are apparently distinct from the dehydratases of the groups described above. Our data indicate that *Saccharopolyspora erythraea* (NRRL2338) is closer related to *S. fradiae* (T59235), the producer of tylosin, than to any other *Streptomyces* strain. Erythromycin and tylosin are both macrolite antibiotics which contain similar sugar moieties.

(iv) *S. viridochromogenes* (Tü57) is producing the oligosaccharide antibiotic avilamycin A containing different deoxysugar moieties than the other strains investigated in this study (Table 1). This might explain why the dehydratase from *S. viridochromogenes* (Tü57) is distinct from the other dehydratases. The function of the dehydratases from *S. cinnamonensis* (Tü89) and *S. olivaceus* (Tü 2353) has not been investigated so far. The phylogenetic analysis might indicate that the dehydratase I of *S. cinnamonensis* (Tü89) is involved in the production of a deoxysugar moiety with a structure similar to D-olivose or L-rhodinose. This sugar might be connected to a polyketide moiety.

3.4 Conclusion

We have developed a general approach for identifying and cloning dehydratase genes from different organisms. Our data indicate that the phylogenic tree constructed from the deduced protein sequences of the isolated PCR fragments correlates with the structure of the deoxysugar moiety of the natural compound produced by the actinomycetes strains. In the rapidly developing field of constructing hybrid antibiotics [19] this method will support the cloning of genes envolved in the biosynthesis of sugar moieties of natural compounds in

different *Actinomycetes* strains and may help to verify the „genetic screening" for unknown substances.

Acknowledgements

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Strains	Known antibiotic with deoxy-sugar moiety produced by the strain	Classification of the antibiotic	Deoxysugar moiety	Reference
Amycolatopsis mediterranei (DSM5908)	balhimycin	glycopeptide	dehydrovancosamin	[20]
S. cinnamonensis (Tü89) fragment 1 fragment 2	kirrothricin	polyene	(not determined)	[21]
S. cyanogenus S136 (DSM 5087)	landomycin A	polyketide	D-olivose; L-rhodinose	[22]
S. fradiae (Tü2717)	urdamycin A	polyketide	D-olivose; L-rhodinose	[23]
S. ghanaensis (ATCC14672)	moenomycin A	phospho-glycolipide	D-chinovosamin; 2-deoxy, 2-amino-D-glucose	[24]
S. glaucescens (DSM 40716)	hydroxy-streptomycin	aminoglycoside	5-hydroxy-L-streptose	[18]
S. olivaceus (Tü 2353)	elloramycin	polyketide	2,3,4-O-methyl-L-rhamnose	[25]
S.virido-chromogenes (Tü57)	avilamycin A	oligoglycoside	2-deoxy-D-rhamnose; 4-O-methyl-L-fucose	[26]

Table 1: Actinomycetes strains which were used for PCR amplification of dNDP-glucose 4,6-dehydratase genes.

S. violaceoruber (1) M--RLLV**TGAAGFIG**SHYVREILAGSYPES-----
S. erythraea (1) M--RVLV**TGGAGFIG**SHYVRQLLGGAYPAF-----
S. griseus (1) MTTHLLV**TGAAGFIG**SQYVRTLLGPGGPP------

consensus sequence: TG(A,G)AGFIG

------HGLDVRITRC **SNNYGPRQHP** EKLIPNFVTR (200) ---
------HGLPVCITRC **SNNYGPYQFP** EKVLPLFITN (200) ---
------HGLDVRVTRC **SNNYGPRQFP** EKLIPRFITL (200) ---

consensus sequence: NNYGP(Y,R)Q(H,F)P

```
                                10        20        30        40        50
A.MEDITERRANEI (DSM5908)      1 SHYVRQVLTG AYP-------- ----KLTYAG NEANLAPVAA DPRLEFVRGD  50
S.CINNAMONENSIS (TU89) I      1 STYVRTLLDG GYPGYEGAEV TVLDKLTYAG NRDNLPA--T EPRMTFVRGD  50
S.CINNAMONENSIS (TU89) II     1 SCPVRLLLGP EAPV-RVTGV TVLDELTYAG NRDNLAPVET DPRLTPVEGD  50
S.FRADIAE (TÜ2717)            1 SHPVRSLLAD TYSGWEGARV TALDKLTYAG NRNNLPP--S NPRLEPVRGD  50
S.CYANOGENUS S136 (DSM5087)   1 RLYVRTLLND GYEDWKGAEV TVLDKLTYAG NRDNLPE--R TRGLTPVQGD  50
S.GLAUCESCENS (DSM40716)      1 SHYVRTLLGP DGPPDAV--V TVLDALSYAG NLANLDPVRD EPRLRPVEGD  50
S.GHANAENSIS (ATCC14672)      1 SHYVRTLLGP QGPGDVA--I TVLDKLTYAG NPANLDEVRA EPGFAPVQGD  50
S.OLIVACEUS (TÜ2353)          1 SQPVRALLSE ELPSGKGAQV TVLDNLTYSG NEANLAPVAD KPGYTPVRGD  50
S.VIRIDOCHROMOGENES (TÜ57)    1 SQYVRELVRD GDP----ARV TVLDKLTYAG NLANLEPVAG --RYTPVEGD  50
CONSENSUS SEQUENCE              SHYVRTLLG  GYPG  GA V TVLDKLTYAG NRANL PVA  EPRLTPVRGD

                                60        70        80        90       100
A.MEDITERRANEI               51 ICDTALVADV MEGVDLVVEF AAESHVDRSI PGAADFVLTN VLGTQNLLQA 100
S.CINNAMONENSIS I            51 INDLPLLLDL LPGHDAVVEF AAESHVDRSL TAAAKFIRTN VCGTQNLLEA 100
S.CINNAMONENSIS II           51 IRDTALAGVA VVESDAVVEF AAESHVDRSI DGAADFVSTN VLGTQTLLDA 100
S.FRADIAE                    51 VCDRALLREL LPGHDAVVEF AAESHVDRSL EGAGEFFRTN VLGTQTLLDA 100
S.CYANOGENUS S136            51 ICDPELLLEL LPGHDAVVEF AAESHVDRSL ESAEEFVETN VTGTQRLLDA 100
S.GLAUCESCENS                51 ICDADLVDRV MAGQDQVVEL AAESHVDRSL LDAAAFVRTN AGGTQTLLDA 100
S.GHANAENSIS                 51 ICDPELVGKL MAEHDQVVEF AAESHVDRSI DGGAEFVRTN VVGTETLIDA 100
S.OLIVACEUS                  51 IRDYAVVDDA MRGQDAVVEF AAESTWERSI LDSSPFVTAE VLGTQVLLDA 100
S.VIRIDOCHROMOGENES          51 ICDARLLAEV VPGHDLVVNF AAESHVDRSI ADAAPFIRTN VQGAENSCR- 100
CONSENSUS SEQUENCE             CD AL   L MPGDHAVVEF AAESHVDRSI  GAAEFVRTN VLGTQTLLDA

                               110       120       130       140       150
A.MEDITERRANEI              101 ALEAGVGKVV HVSTDEVYGS IEHGSWTEDH VLEPNSPYSA SKASSDLVAR 150
S.CINNAMONENSIS I           101 CLRSGVQRVV HVSTDEVYGS IAEGSWTEEW PLEPNTPYAA SKAASDLVAR 150
S.CINNAMONENSIS II          101 ALREGGRTFL HVSTDEVYGS VPEGSWTEEH PLAPNSPYSA SKASSDLIAL 150
S.FRADIAE                   101 VLDSGVERVV HVSTDEVYGS IEQGSWTEDW PLQPNSPYAA SKACSDLVAR 150
S.CYANOGENUS                101 VLATRVKKVV HVSTDEVYGS IDEGSWTEEW PLAPNSPYSA SKAASDLLAR 150
S.GLAUCESCENS               101 ALREGVAPFV QVSTDEVYGS LETGSWTEDE PLRPNSPYAT SKASGDLIAL 150
S.GHANAENSIS                101 AERAGIETFV HISTDEVYGS IDEGSWFETH PLQPNSPYSS AKASSDLIAL 150
S.OLIVACEUS                 101 PKRHGVGRFV HVSTDEVYGS ISEGSWTEDW PLALNSPYSA SKAGSDLLAL 150
S.VIRIDOCHROMOGENES         101 CLEAGTQGIV QVSTDEVYGS IETGSWDEDA PIAPNSPYAA SKAGGDMVAL 150
CONSENSUS SEQUENCE            ALR GV R V HVSTDEVYGS IEEGSWTEDW PIAPNSPYSA SKASSDLVAL

                               160       170       180       190       200
A.MEDITERRANEI              151 SFFRTHGLPV CVTRCS.... .......... .......... .......... 200
S.CINNAMONENSIS I           151 SYWKTHGLDL SITRCS.... .......... .......... .......... 200
S.CINNAMONENSIS II          151 AYERTHGLDV RVTRCS.... .......... .......... .......... 200
S.FRADIAE                   151 AYWRTHGVDL SITRCS.... .......... .......... .......... 200
S.CYANOGENUS S136           151 SYWRTHGLDL SITRCS.... .......... .......... .......... 200
S.GLAUCESCENS               151 AMEVSHGLDV RITRCS.... .......... .......... .......... 200
S.GHANAENSIS                151 SYERTHGLDV RVTRCS.... .......... .......... .......... 200
S.OLIVACEUS                 151 SYERTHGMDV VVTRCS.... .......... .......... .......... 200
S.VIRIDOCHROMOGENES         151 AYARTHGLPV SVTRCG.... .......... .......... .......... 200
CONSENSUS SEQUENCE            SYERTHGLDV SVTRCS
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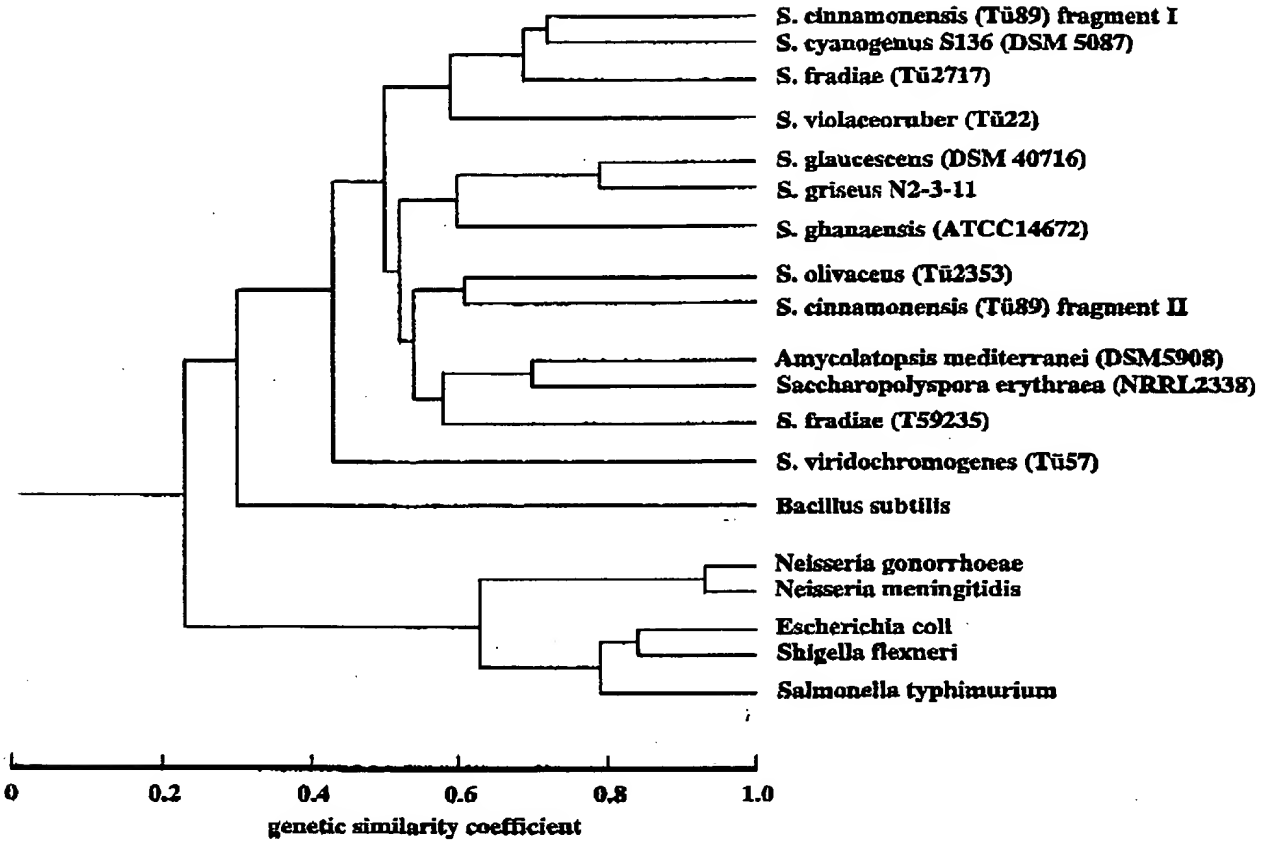
S. cinnamonensis (Tü89) fragment I
S. cyanogenus S136 (DSM 5087)
S. fradiae (Tü2717)
S. violaceoruber (Tü22)
S. glaucescens (DSM 40716)
S. griseus N2-3-11
S. ghanaensis (ATCC14672)
S. olivaceus (Tü2353)
S. cinnamonensis (Tü89) fragment II
Amycolatopsis mediterranei (DSM5908)
Saccharopolyspora erythraea (NRRL2338)
S. fradiae (T59235)
S. viridochromogenes (Tü57)
Bacillus subtilis
Neisseria gonorrhoeae
Neisseria meningitidis
Escherichia coli
Shigella flexneri
Salmonella typhimurium
0
0.2
0.4
0.6
0.8
1.0
genetic similarity coefficient

Figure 1: Homologous regions in the amino acid sequence of the N-terminus of dNDP-glucose dehydratases from *Streptomyces violaceoruber* (Tü22), *Saccharopolyspora erythraea* (NRRL2338) *and Streptomyces griseus N2-3-11*. (The consensus sequences of the amino acid sequence located at position 5-15 and 175-188 (bold letters) of the proteins were used to design oligonucleotide primers for the amplification of dNDP-glucose dehydratase genes from actinomycete species)

Figure 2: Alignment of the derived amino acid sequences of the cloned dehydratase fragments from different actinomycete species. (The fragments were obtained by PCR using oligonucleotide primers designed from the consensus amino acid sequences of different dNDP-glucose dehydratases (e.g.: position 6-15 and 179-187 of StrE from *S. griseus N2-3-11*) . The amino acid sequence encoded by the oligonucleotide primers sequences are not included. Those amino acids present at least in 4 of 9 are defined as consensus sequence.

Figure 3: Phylogenetic tree based on genetic similarities. (The amino acid sequences were taken from the following sources: *S. violaceoruber* (Tü22), amino acid 14-177 (gp L37334); *S. griseus N2-3-11*, amino acid 16-178 (sp P29782); *Saccharopolyspora erythraea* (NRRL2338), amino acid 14-179 (gp L37354); *S. fradiae* (T59235), amino acid 14-179 (gp U08223); *Neisseria gonorrhoeae*, amino acid 20-193 (sp P37761); *Neisseria meningitidis*, amino acid 20-193 (gp L09188); *Escherichia coli*, amino acid 14-195 (gp U23775); *Shigella flexneri*, amino acid 14-195 (sp P37777); *Salmonella typhimurium*, amino acid 14-195 (sp P26391) and *Bacillus subtilis*, amino acid 16-179 (sp P39630); the sequences of all other strains have been determined in this study)

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J Bacteriol. 1999 January; 181 (1): 305–318

Doxorubicin Overproduction in *Streptomyces peucetius*: Cloning and Characterization of the *dnrL* Ketoreductase and *dnrV* Genes and the *doxA* Cytochrome P-450 Hydroxylase Gene

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Abstract

Doxorubicin-overproducing strains of *Streptomyces peucetius* ATCC 29050 can be obtained through manipulation of the genes in the region of the doxorubicin (DXR) gene cluster that contains *dpsH*, the *dpsG* polyketide synthase gene, the putative *dnrU* ketoreductase gene, *dnrV*, and the *doxA* cytochrome P-450 gene. These five genes were characterized by sequence analysis, and the effects of replacing *dnrU*, *dnrV*, *doxA*, or *dpsH* with mutan alleles and of *doxA* overexpression on the production of the principal anthracycline metabolites of *S. peucetius* were studied. The exact roles of *dpsH* and *dnrV* could not be established, although *dnrV* is implicated in the enzymatic reactions catalyzed by DoxA, but *dnrU* appears to encode a ketoreductase specific for the C-13 carbonyl of daunorubicin (DNR) and DXR or their biosynthetic precursors. The highest DXR titers were obtained in a *dnrX dnrU* (N. Lomovskaya, Y. Doi-Katayama, S. Filippini, C. Nastro, L. Fonstein, M. Gallo, A. L. Colombo, and C. R. Hutchinson, J. Bacteriol. 180:2379–2386, 1998) double mutant and a *dnrX dnrU dnrH* (C. Scotti and C. R. Hutchinson, J. Bacteriol. 178:7316–7321, 1996) triple mutant. Overexpression of *doxA* in a *doxA::aphII* mutant resulted in the accumulation of DXR precursors instead of in a notable increase in DXR production. In contrast, overexpression of *dnrV* and *doxA* jointly in the *dnrX dnrU* double mutant or the *dnrX dnrU dnrH* triple mutant increased the

DXR titer 36 to 86%.

1: J Antibiot (Tokyo). 1993 Jan;46(1):56-64. Related Articles

Anthracycline metabolites from baumycin-producing Streptomyces sp. D788. II. New anthracycline metabolites produced by a blocked mutant strain RPM-5.

Yoshimoto A, Fujii S, Johdo O, Kubo K, Nishida H, Okamoto R, Takeuchi T.

Central Research Laboratories, Mercian Corporation, Kanagawa, Japan.

A daunorubicin-blocked mutant strain RPM-5 derived from a new baumy producing Streptomyces sp. D788 accumulated a major precursor metabo D788-1 (10-carboxyl-13-deoxocarminomycin) and nine minor metabolite the culture broth. Five among them were new with a substituent at C-10 c altered side chains at C-9. Isolation, purification and identification of all anthracycline metabolites produced by strain RPM-5 are described with t antitumor activities against L1210 cells.

MeSH Terms:

- Animals
- Antibiotics, Antineoplastic/chemistry
- Antibiotics, Antineoplastic/isolation & purification*
- Antibiotics, Antineoplastic/pharmacology*
- Carubicin/analogs & derivatives
- Carubicin/chemistry
- Carubicin/isolation & purification
- Carubicin/pharmacology
- DNA Replication/drug effects
- DNA, Neoplasm/drug effects
- Daunorubicin/analogs & derivatives*
- Daunorubicin/biosynthesis
- Daunorubicin/pharmacology
- Fermentation
- Leukemia L1210/drug therapy
- Magnetic Resonance Spectroscopy
- Mice
- RNA, Neoplasm/drug effects
- Streptomyces/chemistry*
- Streptomyces/drug effects
- Tumor Cells, Cultured

Substances:

- Antibiotics, Antineoplastic

- DNA, Neoplasm
- RNA, Neoplasm
- Daunorubicin
- Carubicin
- baumycins
- 10-carboxy-13-deoxocarminomycin

PMID: 8436560 [PubMed - indexed for MEDLINE]

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Journal of Bacteriology

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J Bacteriol. 1986 August; 167 (2): 581–586

Anthracycline metabolites of tetracenomycin C-nonproducing Streptomyces glaucescens mutants.

S Yue, H Motamedi, E Wendt-Pienkowski, and C R Hutchinson

Abstract

Mutants of Streptomyces glaucescens GLA.0 which are blocked in the production of tetracenomycin C (compound 1), an anthracycline antibiotic having significant antitumor activity, accumulated several new anthracycline metabolites structurally related to compound 1 and to intermediates of its biosynthetic pathway. Through chemical and spectroscopic comparisons with the known anthracycline metabolites of the wild-type strain, we identified the two regioisomers of tetracenomycin B2 (compounds 7a and 7b), 8-demethyltetracenomycin C (compound 12), tetracenomycin D2 (compound 11), tetracenomycin E (compound 13), and the 12-naphthacenone forms of compounds 7a, 7b, and 2 (tetracenomycin D1). A hypothetical biosynthetic pathway to compound 1 is presented that is consistent with the occurrence of compounds 7b, 13, and 5 (tetracenomycin A2) and with the cosynthetic behavior of tetracenomycin C-nonproducing mutants (H. Motamedi, E. Wendt-Pienkowski, and C. R. Hutchinson, J. Bacteriol. 167:575-580, 1986).

Full text

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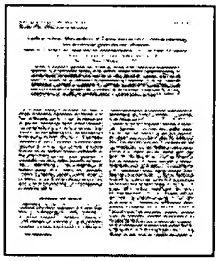
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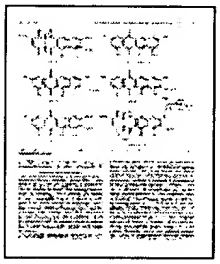
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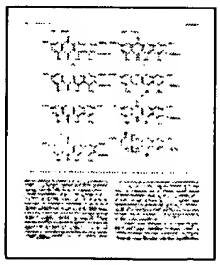
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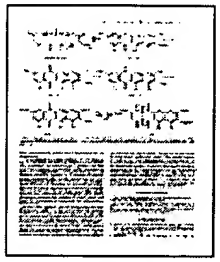

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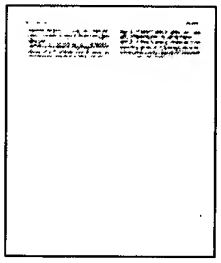

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Mol Gen Genet (1996) 251:113–120

ORIGINAL PAPER

Kristiina Ylihonko · Jaana Tuikkanen · Sanna Jussila
Lina Cong · P. Mäntsälä

A gene cluster involved in nogalamycin biosynthesis from *Streptomyces nogalater*: sequence analysis and complementation of early-block mutations in the anthracycline pathway

Received: 28 November 1995 / Accepted: 23 January 1996

Abstract We have analyzed an anthracycline biosynthesis gene cluster from *Streptomyces nogalater*. Based on sequence analysis, a contiguous region of 11 kb is deduced to include genes for the early steps in anthracycline biosynthesis, a regulatory gene (*snoA*) promoting the expression of the biosynthetic genes, and at least one gene whose product might have a role in modification of the glycoside moiety. The three ORFs encoding a minimal polyketide synthase (PKS) are separated from the regulatory gene (*snoA*) by a comparatively AT-rich region (GC content 60%). Subfragments of the DNA region were transferred to *Streptomyces galilaeus* mutants blocked in aclacinomycin biosynthesis, and to a regulatory mutant of *S. nogalater*. The *S. galilaeus* mutants carrying the *S. nogalater* minimal PKS genes produced auramycinone glycosides, demonstrating replacement of the starter unit for polyketide biosynthesis. The product of *snoA* seems to be needed for expression of at least the genes for the minimal PKS.

Key words *Streptomyces* · Nogalamycin · Anthracycline · Polyketide synthase

Introduction

Polyketides form a large and highly variable group of secondary metabolites produced mainly by bacteria, fungi and plants. Many of them have useful properties as antibiotics, chemotherapeutic agents (anthracyclines), antiparasitics or insecticides. The biosynthetic pathway for aromatic polyketides starts from a simple carboxylic acid residue, to which acetate units are added to build the growing polyketide chain. A multienzyme polyketide synthase (PKS) is responsible for forming a hypothetical linear or monocyclic structure, the polyketide backbone, which is released from the enzyme complex and subsequently modified in various ways. PKS gene clusters are highly conserved; this offers a way to identify polyketide genes from different polyketide producers.

The anthracyclines produced by *Streptomyces* spp. form a group of clinically useful antitumour agents. Their biosynthesis proceeds from a linear decaketide to the aglycone moiety, to which sugar residues are then attached. The pathway to the aglycone moiety of an anthracycline is shown in Fig. 1. A common precursor for most natural anthracyclines is aklavinone, which undergoes glycosidations and modifications, such as hydroxylations and methylations, to form a large variety of anthracyclines. In the biosynthesis of aclacinomycines (Oki et al. 1975) three sugar residues are added to aklavinone without any modification of the aglycone skeleton. Nogalamycin (Wiley et al. 1968) differs from aclacinomycin in its glycosylation profile as well as in the aglycone moiety. The aglycone of nogalamycin is made from ten acetate units, whereas one propionate starter and nine acetates are used to synthesize aklavinone. Another difference between the aglycones is in the opposite stereochemistry at C9. Auramycinone, like the aglycone of nogalamycin, is formed from ten acetates but the configuration at C9 is the same as that in aklavinone (Fujiwara et al. 1981).

We have recently cloned a 12 kb *Bgl*II fragment (in pSY15) containing a region of the *S. nogalater* genomic DNA that hybridises with the actinorhodin minimal PKS genes, *act*I (Malpartida et al. 1987). The cloned genes were implicated in anthracycline biosynthesis by structural determination of the compounds produced in heterologous hosts (Ylihonko et al., unpublished).

Communicated by A. Kondorosi

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Arcanum, Vatselantie 2, FIN-20500 Turku, Finland

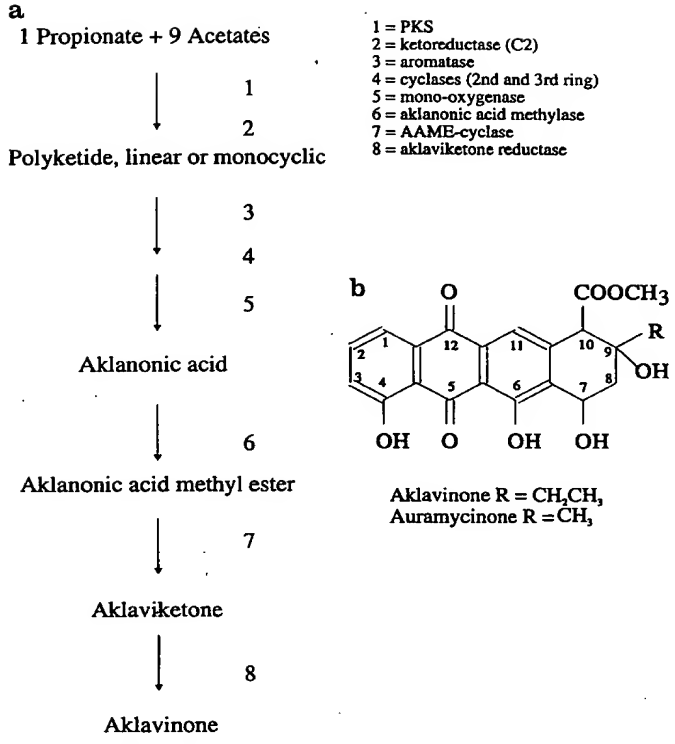

Fig. 1 **a** Proposed steps in biosynthesis of aklavinone. The biosynthetic steps leading to auramycinone are probably analogous except that synthesis begins with an acetate starter unit. **b** Structures of aklavinone and auramycinone

Here, we describe the structure and deduced functions of the gene cluster encoding the nogalamycin minimal PKS and its surrounding DNA. The DNA fragments from the region were subcloned into non-producer *S. galilaeus* and *S. nogalater* mutants in order to characterize the mutated biosynthetic steps by functional complementation.

Materials and methods

Microbial strains and plasmids

Manipulations of *S. nogalater* DNA were carried out in *Escherichia coli* XL1-Blue (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac* [F′ *proAB*, *lacI*^{q}*ZM15*, Tn*10* (Tet^r)] (Stratagene, La Jolla, Calif.). *Streptomyces* strains used were *S. nogalater* ATCC 27451, the blocked *S. nogalater* mutant *Sno*-615 (Cong et al., unpublished), *S. galilaeus* ATCC 31615 (Fujiwara et al. 1980), the blocked *S. galileus* mutants H039, H028, H071 and H061 (Ylihonko et al. 1994), and *S. lividans* TK24 (Hopwood et al. 1985). The plasmids used are listed in Table 1.

Culture conditions and cloning procedures

Streptomyces spp. were transformed by the standard method (Hopwood et al. 1985) with minor modifications. DNA propagated in *E. coli* was ligated into pIJ486 and introduced by transformation into *S. lividans* TK24. Plasmid constructions were then isolated from TK24 and introduced into *S. galilaeus* or *S. nogalater*. Transformation frequencies (transformants/μg DNA) with plasmids isolated from TK24 were about 10^6 for TK24, 1 for *S. galilaeus* and 10^{-1} for *S. nogalater*. DNA isolation and manipulations were carried out by standard procedures (Sambrook et al. 1989; Hopwood et al. 1985).

Sequencing and sequence analysis

The Wizard Miniprep DNA Purification System (Promega Madison, Wis.) was used for isolating plasmids from *E. coli*. DNA sequencing was performed using the Deaza G/A T7 sequencing kit from Pharmacia (Sweden) and the TaqTrack Deaza system of Promega, according to the manufacturers' instructions. In sequencing reactions, deazaG and deazaA and an annealing temperature of 45° C instead of 37° C were used to alleviate problems of compression caused by the high GC content of DNA. Sequence analyses were done with the GCG sequence analysis software package (University of Wisconsin Genetics Computer Group programs). The translation table was modified to accept GTG also as a start codon. Codon usage was analysed using published data (Wright and Bibb 1992).

Table 1 Plasmids used

Plasmid	Relevant characteristics	Reference or source
pUC18/19	*E. coli* plasmid	Yanisch-Perron et al. (1985)
pIJ486	*Streptomyces* plasmid	Ward et al. (1986)
pIJE486	*ermE* (Bibb et al. 1985) cloned into polylinker of pIJ486	This work
pSY15[a] (1-11)	Causes the production of the nogalamycin chromophore	Ylihonko et al. (unpublished)
pSY1[a] (7-10)	Hybridizes with *act*I	Ylihonko et al. (unpublished)
pSY3[a] (10-13)	*sno*X-Y	This work
pSY21[a] (4-11)	*sno*A-1-2-3	This work
pSYE181[a] (8-11)	*sno*1-2-3[b]	This work
pSY18[a] (7-11)	*sno*1-2-3	This work
pSY22[a] (5-7)	*sno*A^{415}(lacks from C-terminal 250 aa)	This work
pSY24[a] (2-3)	*sno*D[b]	This work

[a] The inserts in each pSY plasmid are indicated by the numbers in parentheses, which correspond to the restriction sites
[b] The fragment is cloned in pIJE486

Expression constructs and complementation of *S. galilaeus* mutants

Expression constructs were made by subcloning fragments of the sequenced gene cluster, inserted in the polylinker of pIJ486 or pIJE486, into TK24. After isolation from TK24, the plasmids were transferred into the *S. galilaeus* mutants and into *Sno*-615 to study complementation. The transformants were plated on ISP4 agar supplemented with thiostrepton (50 μg/ml) and the colonies were used to inoculate anthracycline production medium E1 (Ylihonko et al. 1994). Anthracycline production was assessed by extracting a sample of E1 culture medium with organic solvents and analysing the extract by thin layer chromatography (TLC) as described (Ylihonko et al. 1994). The aglycone moiety was determined, after hydrolysis of the E1 culture medium in 1 M HCl for 30 min at 80° C, by TLC, using aklavinone and auramycinone (kindly provided by Galilaeus Oy, Turku, Finland) as standards. For quantitative analysis, HPLC was performed on a octadecyl reverse phase column (RP-18) with a mobile phase of acetonitrile: 60 mM KH_2PO_4 (pH 3) 7:3 and detection at 254 nm.

Results

Cloning and sequencing of PKS genes

The *act*I genes from pIJ2345 (Malpartida et al. 1987) and *acm* from pACM5 (Niemi et al. 1994) were used as probes to screen for anthracycline biosynthetic genes in a library of *S. nogalater* DNA. Hybridization with *act*I revealed 2-kb (pSY1) and 3.8-kb (pSY3) *Eco*RI fragments and the 12-kb *Bgl*II fragment (pSY15) was detected by both *act*I and *acm* (Fig 2). pSY15 caused the production of anthracycline metabolites in *S. lividans* (Ylihonko et al., unpublished). Sequenced regions (pSY3 and 9 kb of the 12-kb pSY15 insert) are shown in bold in Fig 2.

Sixty subclones were made, using convenient restriction sites, to facilitate sequencing of both strands of the 11-kb DNA region. Nine complete (ORF1, 2, 3, A, B, C, D, X, Y) and two incomplete (ORFE, Z) *sno* open reading frames were found. The criteria for revealing ORFs were a typical codon usage, as detected by the CODONPREFERENCE program, and identification of a possible ribosome-binding site (RBS) complementary to the 3′ end of *S. lividans* 16S rRNA (Bibb and Cohen 1982) about 10 bp upstream of each potential translational start codon. Possible functions of the genes were deduced from the results of a computer-aided homology search using the FASTA and TFASTA programs. The nucleotide sequence has been submitted to the EMBL Database under the accession number Z48262.

Deduced functions of the *sno* ORFs

1. *Minimal PKS.* The minimal PKS is responsible for the formation of a hypothetical polyketide structure which, most probably, is either linear or monocyclic (McDaniel et al. 1994a). Three conserved ORFs (*sno*1, *sno*2, *sno*3) of the Type II PKS have so far almost always been found in the same order in polyketide gene clusters (the ACP gene lies elsewhere in *S. peucetius*; Grimm et al. 1994). The products of these genes are thought to be: (1) ketosynthase (KS) with possible acyltransferase (AT) activity; (2) a chain length determining factor (CLF); and (3) acyl carrier protein (ACP) (McDaniel et al.1994a). The *sno*1 and *sno*2 ORFs are translationally coupled, as in nearly all other type II PKS clusters, with a 4-bp overlap *sno*1 encodes a peptide of 430 amino acids with a high degree of similarity to the ketoacyl synthases of other aromatic PKSs (Table 2). *sno*2 encodes a 409-amino acid peptide. The similarity between Sno2 and Sno1 is about 50%; this is a common feature of *Streptomyces* aromatic PKS genes. The products of these two ORFs are assumed to be subunits of the PKS complex; the active centers of both KS and AT are missing in Sno2. Sno2 is proposed to act as a chain length determining factor (McDaniel et al. 1993). As in other PKS clusters, a small *sno*3 ORF encoding an 86-amino acid peptide follows downstream from *sno*2; 59 b separate *sno*2 from *sno*3. On the basis of its sequence, Sno3 is an acyl carrier protein.
2. *sno*A. The *sno*A gene, upstream of the minimal PKS genes, encodes a peptide of 665 amino acids. Its deduced product exhibits high similarity to the products of *S. peucetius* and *S. coelicolor* genes that have been implicated in the regulation of secondary metabolism

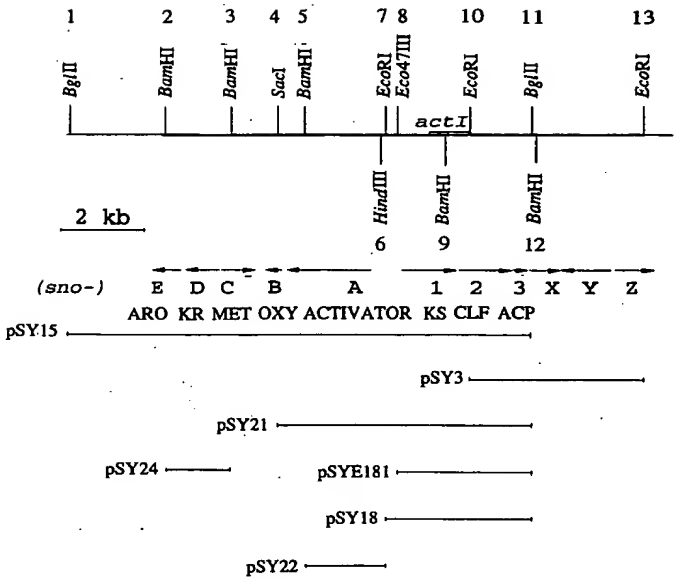

Fig. 2 Restriction map of the PKS cluster from *S. nogalater*. The ORFs revealed by CODONPREFERENCE and the direction of transcription are shown by *arrows*. The region that hybridizes with *act*I (Malpartida et al. 1987) is indicated. The numbering of the restriction sites used for subcloning corresponds to that in Table 1. The sequenced region is marked as a *thick line*. Corresponding DNA fragments for pSYclones are indicated. Abbreviations of ORF functions are: ARO, aromatase; KR, ketoreductase, MET, methyl transferase; OXY, mono-oxygenase; KS, ketosynthase; CLF, chain length determining factor; ACP, acyl carrier protein

Table 2 Closest similarities and predicted functions of *sno-orf* products deduced from sequence comparisions

ORF product	Length (aa)	Deduced function	Degree of identity (%) and antibiotic cluster	Reference
SnoA	665	Regulatory	DnrI (30.7) Daunomycin	Stutzman-Engwall et al. (1992)
SnoB	119	Mono-oxygenase	ORF8 (47.0) Daunomycin	Grimm et al. (1994)
SnoC	270	Aklanonic acid methyl transferase	DauC (53.0) Daunomycin	Dickens et al. (1995)
SnoD	262	Ketoreductase	AknIII (73.0) Aclacinomycin	Tsukamoto et al. (1992)
SnoE[a]		Aromatase	DauA (48.0) Daunomycin	Ye et al. (1994)
Sno1	430	KS, AT	Gra (73.6) Granaticin	Sherman et al. (1989)
Sno2	409	CLF	Otc (70.6) Oxytetracycline	Kim et al. (1994)
Sno3	86	ACP	Otc (67.5) Oxytetracycline	Kim et al. (1994)
SnoX	246	Methylase	RdmD (61.0) Rhodomycin	Niemi and Mäntsälä (1995)

[a] This sequence is incomplete

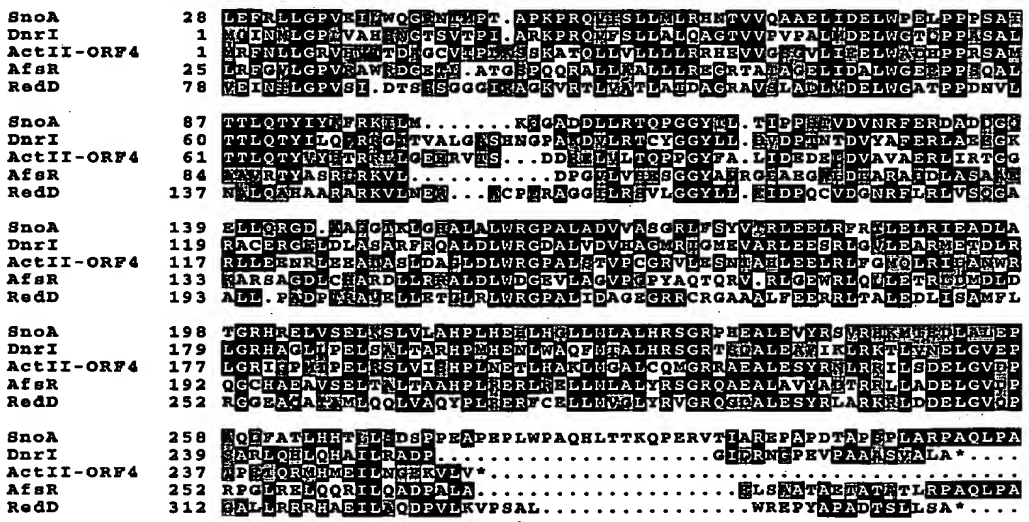

Fig. 3 Alignment of amino acid sequences (PILEUP) of four different regulators: SnoA from *S. nogalater* (Z48262); DnrI from *S. peucetius* (M80237); AfsR (D90155), RedD (M29790) and ActII-ORF4 (M64683) from *S. coelicolor*. The sequences have been arranged in rank order of pairwise similarity to SnoA

(Stutzman-Engwall et al. 1992; Stein and Cohen 1989). The similarity/identity of SnoA to the products of such regulatory genes ranges from 63%/43% for *Dnr*I (Stutzman-Engwall et al. 1992); 63%/39% for ActII-ORF4 (Fernandez-Moreno et al. 1991); 58%/39% for AfsR (Horinouchi et al. 1990) and 51%/33% for RedD (Narva and Feitelson 1990), respectively: the alignment of the regulatory genes (Fig. 3) revealed high conservation. Three of these activators (DnrI, ActII-ORF4 and RedD) are each only about 300 amino acids long and are similar to the N-terminal portions of the longer peptides SnoA (665 residues) and AfsR (995), while the whole sequence of SnoA is homologous to the N-terminal half of AfsR.

*sno*A is transcribed in the opposite direction from the genes for the minimal PKS and an AT-rich region of 756 bp separates them (Fig. 4). The GC content of the entire sequenced region is 71%, but in the region between *sno*A and the first ORF of the minimal PKS (*sno*1) it is only 60%. Based on its low GC content and the presence of inverted repeats (Fig. 4), the region may be involved in regulation of anthracyline biosynthesis. 3. *sno*B, *sno*C, *sno*D and *sno*E. The protein coding regions (Fig. 2) found in pSY15 encode the biosynthetic enzymes needed for early steps in the biosynthesis of the anthracyclines. The similarities to the daunomycin biosynthetic genes (Ye et al. 1994; Dickens et al. 1995; Grimm et al. 1994) are remarkable and the gene

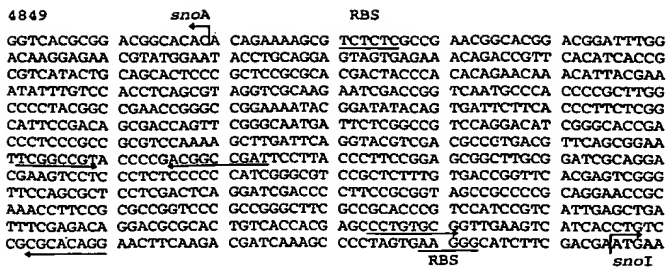

Fig. 4 Sequence of an AT-rich region that separates the minimal PKS ORFs from the start codon of the regulatory gene. Inverted repeats are shown by *thin arrows*

functions suggested here are mainly based on this comparison. SnoE resembles aromatases, enzymes proposed to be responsible for making the first ring aromatic in type II polyketides (McDaniel et al. 1994b) (step 3, Fig. 1). Only 134 amino acids of the N-terminal part are included in the sequenced region, although the whole ORF was apparently present in pSY15. *sno*D encodes a 262-amino acid peptide, which shows similarity to ketoreductases of polyketides, which catalyse the region-specific ketoreduction of the nascent polyketide chain (Fu et al. 1994). Mutations in such a ketoreductase lead to the formation of 2-OH anthracyclines or their intermediates (Matsuzawa et al. 1981; Ylihonko et al. 1994). *sno*C, which is transcribed in the opposite direction to the other three ORFs, encodes a peptide of 292 amino acids. Based on homology to *dau*C (Dickens et al. 1995), the proposed function of SnoC is methylation of aklanonic acid (step 6, Fig. 1). *snoB* encodes a peptide of 119 amino acids, which probably oxygenates C12 (Summers et al. 1993) (step 5, Fig. 1).

4. *sno*X and *sno*Y. *sno*X, downstream from *sno*3, is remarkably similar to *srm*X from *S. ambofaciens* (Geistlich et al. 1992) and to *rdm*D from *S. purpurascens* (Niemi and Mäntsälä 1995). The similarity at the amino acid level was 69% and 71%, respectively. A computer-aided PROFILE SEARCH of the multiple sequence alignment of SrmX, RdmD and SnoX found methylases. The gene product of *rdm*D may be involved in N,N-dimethylation of daunosamine to make rhodosamine, the first sugar residue attached to the aglycone moiety in rhodomycin. The sugar residue at C1-C2 of nogalamycin, nogalamine, also contains a N,N-dimethyl group. *sno*X is closely linked to the minimal PKS genes, while *rdm*D is in the same cluster as the ORFs needed for the last steps of rhodomycine biosynthesis, the genes responsible for aklavinone modifications. *sno*X encodes a peptide of 246 amino acids.

The last complete ORF (*sno*Y) in the sequenced region is transcribed in the opposite direction. The deduced amino acid sequence did not closely resemble that of any protein in known polyketide clusters and the similarities to other gene products were not significant.

Expression of *sno* genes in *S. galilaeus* and in *S. nogalater*

The analysis of early blocked *S. galilaeus* mutants was carried out by using the *sno* genes from *S. nogalater*. The properties of the mutants are shown in Table 3 and the plasmid constructions used for the complementation analysis are listed in Table 1. The products obtained from the recombinant strains were hydrolyzed and the aglycones were determined as described above. Aklavinone and auramycinone differ only in the substituent at C9, which is an ethyl or a methyl group, respectively. Building of the carbon chain begins at C9, suggesting that the starter unit in *S. galilaeus* is different from that in *S. nogalater*. Expression of the nogalamycin minimal PKS was analyzed by following the formation of auramycinone, although auramycinone is not a precursor of nogalamycin because of the opposite stereochemistry of the C9-C10 bond; the stereochemistry of the hybrid products was derived from *S. galilaeus* (Ylihonko et al., unpublished).

The mutant H039 was used as a host in the cloning procedures because it is more easily transformed than the other mutants or the wild type *S. galilaeus*. H039 differs from the wild-type only in the glycosylation profile of aklavinone and we used it to determine the minimal construct that could cause production of auramycinone. Using constructs lacking the minimal PKS region (*sno*1, 2, 3), only aklavinone was obtained, as expected. pSY18, carrying all the minimal PKS genes with its own promoter, was the smallest subclone

Table 3 Properties of the mutants used in the study

Mutant	Product	Complementation[a]	Possible lesion
H039	Akv-rho-rho[b]	pSY18	Glycosylation
H028	Nonproducing	pSYE181	Regulatory
H071	Nonproducing	pSY18	Minimal PKS
H061	2-OH-aklanonic acid	pSY24	Ketoreductase
Sno-615	Nonproducing	pSY22 (nogalamycin)	Regulatory

[a] The smallest construction capable of causing production of auramycinone
[b] Aklavinone-rhodinose-rhodinose

able to cause production of auramycinone. The main product, however, was aklavinone; only 12% was auramycinone. The proportion of auramycinone increased to 29% when the promoter used for the minimal PKS was the constitutively active *erm*E promoter, and to 39% when *sno*A was included.

Previously we observed that pSY15 caused production of auramycinone glycosides in the non-producing *S. galilaeus* mutant H028 (Ylihonko et al., unpublished). The subclones derived from pSY15 indicated that both the minimal PKS genes and *sno*A (pSY21) were needed for production of anthracyclines in H028. pSY18, which caused production of auramycinone in H039, failed to complement H028, but pSYE181 did complement H028. When the products of the culture were hydrolysed, only auramycinone was obtained. Based on the ability of the strain to glycosylate aklavinone we have proposed that H028 has a mutation in a structural gene encoding an early biosynthetic step (Ylihonko et al. 1994). However, the complementation by pSY21 suggested that the mutation is more likely to lie in a regulatory region. The failure of H028/pSY21 to produce aklavinone could have several explanations but the simplest is that H028 has a mutation in the regulatory gene corresponding to *sno*A (or in the promoter of the minimal PKS) and the specificity of SnoA restricts expression of the minimal PKS genes for aklavinone, or, alternatively, the mutation occurred in the promoter region of the minimal PKS genes.

Starting from the hypothesis that the regulatory gene of *S. nogalater* does not activate the *S. galilaeus* PKS genes, pSY22, carrying a 415-residue N-terminal portion of the regulatory gene (*sno*A^{415}), was transferred to wild-type *S. galilaeus* and *S. nogalater*. As expected, pSY22 had no effect on the product profile of *S. galilaeus*, while it increased production of nogalamycin slightly when introduced into wild-type *S. nogalater*.

Sno-615 (Cong et al., unpublished), which was isolated as a non-producing mutant strain after random mutagenesis of *S. nogalater*, cannot produce any colo ured anthracycline intermediates. The failure to cosynthesize nogalamycines with other *sno* mutants suggested that the lesion is in a regulatory gene rather than in a structural gene. In agreement with this pSY22, carrying *sno*A^{415}, was sufficient to complement the mutant. The amount of nogalamycin obtained was equal to that produced by the wild-type *S. nogalater*.

Previously we proposed that H071 has a mutation in an early structural gene of aclacinomycin biosynthesis, because it can convert biosynthetic intermediates to aklavinone glycosides and can cosynthesize with the blocked mutants (Ylihonko et al. 1994). H071 appeared to have a mutation in one of the minimal PKS genes because pSY18, carrying the genes encoding the minimal PKS, restored anthracycline production to H071, and both aklavinone and auramycinone were obtained.

The mutant H061 accumulates 2-OH-aklanonic acid. Complementation with pSY24, carrying *sno*D, was successful and anthracyclines with aklavinone as the aglycone moiety were obtained.

Discussion

Sequence analysis demonstrated that pSY15 carries polyketide biosynthetic genes for a minimal PKS, ketoreductase and aromatase and a possible "post polyketide" gene for oxygenase. The gene cluster involved in biosynthesis of nogalamycin offered the means to characterize further the previously described *S. galilaeus* mutants by screening for heterologous complementation. The complementation experiments confirmed that H071 is defective in the minimal PKS and H061 is defective in ketoreductase. H028 was shown to be a regulatory mutant. The minimal PKS determines the starter unit used in polyketide biosynthesis, as demonstrated by auramycinone production in H039/pSY18 and H071/pSY18.

The *S. galilaeus* mutants H036 (Ylihonko et al. 1994) and H055 (unpublished) produce anthracycline metabolites, spectral analysis of which has revealed them to be aromatic tricyclic structures. These mutants were not complemented by pSY15, suggesting that a cyclase and/or C7 ketoreductase is lacking (H036 was complemented by a putative aklaviketone reductase for daunomycin; our unpublished results). Structural analysis of TK24/pSY15 products (Ylihonko et al., unpublished) also revealed tricyclic structures, thus supporting the hypothesis. In order to find the missing cyclase we sequenced about a 2 kb DNA fragment downstream from the minimal PKS (cyclase genes in many aromatic polyketide clusters lie distal to the minimal PKS cluster). However, the gene products of *sno*X and *sno*Y obtained from the region do not resemble amino acid sequences of cyclases in the database.

We propose that the mutation in H028 occurs in the promoter region for the minimal PKS genes, rather than in a regulatory gene for the biosynthetic genes. The following observations support this conclusion: (i) pSY18, containing only the minimal PKS genes without the regulatory gene, does not complement H028 but does complement H071; (ii) a small quantity of aklavinone is produced in H071/pSYE181 but not in H028/pSYE181, and (iii) differences in the sugar moiety exist between the products of H028/pSY15 and wild-type *S. galilaeus*. SnoX has been proposed to function in modifying the sugar moiety in *S. nogalater*, although no experimental data supporting this suggestion are available. The distance between the stop codon of the last minimal PKS gene and *sno*X is only 32 bp and they are probably transcribed from the same promoter. If the corresponding promoter controls

expression of a set of genes including the minimal PKS genes and a gene affecting glycosylation in *S. galilaeus*, as in *S. nogalater*, it could also explain the change in sugar moiety in the H028/pSY15 product. However, genetic analysis of the mutant H028 is needed to confirm the mutation.

SnoA seems to be crucial for nogalamycin synthesis. *Sno*-615 does not produce detectable amounts of nogalamycin or coloured anthracycline intermediates in the production medium, but can be complemented by *sno*A[415]. Based on sequence analysis, SnoA is undoubtedly a member of a group of regulators including RedD and N-terminus of AfsR. However, the mechanism of regulation is not clear.

Most probably, the promoters of the biosynthetic genes (at least the minimal PKS) need SnoA for expression in *S. nogalater*; in *S. galilaeus* (H039 and H071) expression is promoted by the corresponding protein for aclacinomycin biosynthesis. *dnr*I, the regulatory gene cloned from *S. peucetius*, complements an *act*II-ORF4 mutant (Stutzman-Engwall et al. 1992), thus demonstrating the possibility of promoting expression of PKS genes by heterologous regulator elements. The failure to increase anthracycline production in *S. galilaeus* carrying *sno*A does not exclude the possibility of activation of the PKS genes and the accumulation of unstable or nonconjugated intermediates not detectable in the conditions used to produce and isolate the anthracycline metabolites.

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Purification and characterization of the DNA-binding protein DnrI, a transcriptional factor of daunorubicin biosynthesis in *Streptomyces peucetius*

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Summary

The DnrI protein, essential for the biosynthesis of daunorubicin in *Streptomyces peucetius*, was purified almost to homogeneity from *dnrI* expression strains of *Escherichia coli* and *S. peucetius* through several steps of chromatography. The proteins purified from both organisms had identical chromatographic and electrophoretic behaviour. Purified His-tagged or native DnrI was used to conduct DNA-binding assays by gel mobility-shift analysis, and the results showed no significant difference in the DNA-binding activity of native or His-tagged proteins. DnrI binds specifically to DNA segments containing the intergenic regions separating the putative *dnrG–dpsABCD* and *dpsEF* operons, and the *dnrC* gene and *dnrDKPSQ* operon. DNase I footprinting assays indicated that the DNA-binding sites for DnrI extended from upstream of the −10 to −35 regions of the *dnrG* or *dpsE* promoters to include about 65 bp of the *dnrG–dpsE* intergenic region and about 80 bp of the *dnrC–dnrD* intergenic region. Both binding sites contain imperfect inverted repeat sequences of 6–10 bp with a 5′-TCGAG-3′ consensus sequence that was present in 4 out of 10 other promoter regions in the cluster of daunorubicin biosynthesis genes.

Introduction

Interest in the molecular basis of the regulation of antibiotic biosynthesis has gradually expanded since the production genes were first cloned from *Streptomyces* spp. (Chater and Bibb, 1996). Genes specifically involved in the production of a particular antibiotic are invariably clustered and seem to be organized into several transcription units of varying complexity. Pathway-specific, positively acting genes that are required for the activation of their cognate biosynthetic structural genes have been identified in several of the clusters, such as *redD* for the undecylprodigiosin (RED) (Narva and Feitelson, 1990) and *actII–ORF4* for the actinorhodin (ACT) (Fernandez-Moreno *et al.*, 1991) biosynthesis pathways in *Streptomyces coelicolor*.

We have been engaged in a study of the molecular genetics of daunorubicin (DNR) and doxorubicin (DXR) biosynthesis in *Streptomyces peucetius*. Since DNR and DXR are commercially important cancer chemotherapy drugs, information about the regulation of their biosynthesis could be used to increase DNR or DXR production. Previous reports have demonstrated that DNR biosynthesis is initiated by a type II polyketide synthase (dps) from one propionyl-CoA and nine malonyl-CoA precursor units to produce a decaketide that is converted to aklanonic acid (Hutchinson, 1995) (Fig. 1). The *dpsABCDEFG* and *dnrG* genes (Grimm *et al.*, 1994) govern this process (Fig. 1B). Two other genes (*dnrCD*) code for pathway enzymes that convert aklanonic acid to aklaviketone (Madduri and Hutchinson, 1995a) (Fig. 1A). Homologues of these 10 genes are present in *Streptomyces* sp. strain C5 with the same gene organization as in *S. peucetius* (Dickens *et al.*, 1995; Ye *et al.*, 1994). The *S. peucetius dnr* gene cluster also contains two regulatory genes, *dnrI* and *dnrN*, that control expression of the *dnr* structural and resistance genes (Stutzman-Engwall *et al.*, 1992; Madduri and Hutchinson, 1995b; Otten *et al.*, 1995). Insertional inactivation of *dnrI* blocks the production of DNR and its biosynthetic intermediates like ϵ-rhodomycinone (RHO) (Stutzman-Engwall *et al.*, 1992; Madduri and Hutchinson, 1995b) and prevents transcription of the putative operons containing the *dnrLM*, *dnrXY*, *dnrZUV*, *dpsABCD* and *dnrDKPQS* biosynthesis genes and the *drrAB* and *drrC* resistance genes (Fig. 1B) (Madduri and Hutchinson, 1995b). A *dnrN* mutation causes the same phenotype as the *dnrI* mutation because it is epistatic to *dnrI* (Otten *et al.*, 1995). These results support the conclusion that the *dnrI* gene is involved in the transcriptional activation of DNR biosynthesis genes and show that the *dnrN* gene is

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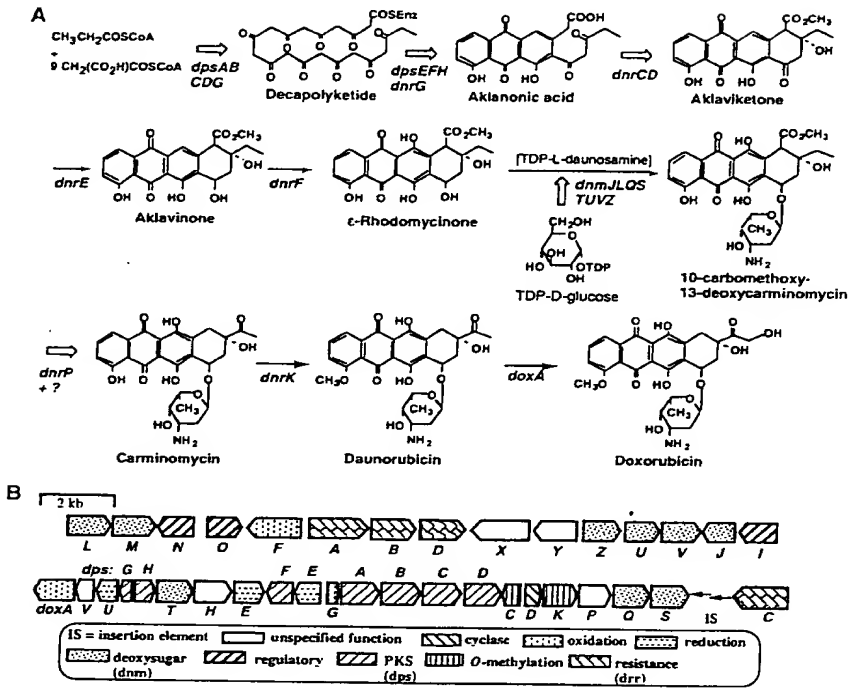

Fig. 1. A. Doxorubicin biosynthesis pathway in *S. peucetius*.
B. Functional map of the *dnr* gene cluster. Some of the gene designations have been modified from Madduri and Hutchinson (1995b) (the daunosamine biosynthesis genes are now named '*dnm*' and all the polyketide synthase genes are named '*dps*') to provide a consistent nomenclature for the genes from *S. peucetius* and *Streptomyces* sp. strain C5 (Dickens *et al.*, 1995; Ye *et al.*, 1994).

required for expression of *dnrI* (Madduri and Hutchinson, 1995b).

As the sequence of DnrI is very similar to the deduced products of the *redD* (Narva and Feitelson, 1990) and *actII–ORF4* (Fernandez-Moreno *et al.*, 1991) transcriptional activators and the N-terminus of the AfsR protein (excluding the ATP and DNA binding motifs in the C-terminus region; Horinouchi *et al.*, 1990) (Stutzman-Engwall *et al.*, 1992), we speculated that the mechanism of transcriptional activation is similar for all of these proteins. As the absence of their function is correlated with the lack of metabolite production and transcription of the biosynthetic and resistance genes in the cases examined, it is likely that DnrI and its close relatives are transcriptional factors that help the organism co-ordinate the expression of antibiotic biosynthesis genes and developmentally control antibiotic production. The *S. coelicolor afsR* gene (Horinouchi *et al.*, 1990) stimulates ACT production in *S. coelicolor* and *Streptomyces lividans* (Stein and Cohen, 1989; Horinouchi *et al.*, 1990). Both the C- and N-terminal portions of AfsR are capable of enhancing pigment production in *S. lividans*, although the C-terminal portion that has the ATP- and DNA-binding motifs is much more effective (Horinouchi *et al.*, 1990). Floriano and Bibb (1996) have recently shown that *afsR* influences pigment production only under some nutritional conditions (primarily as a function of the phosphate concentration) and that expression of *actII–ORF4* and *redD* are not always dependent upon *afsR*. The *dnrI* gene can complement an *actII–ORF4* mutation (Stutzman-Engwall *et al.*, 1992), but *redD* and *actII–ORF4* do not show cross-complementation. Although alignment of the amino acid sequences failed to reveal likely helix-turn-helix DNA-binding motifs in these proteins, except for the C-terminal half of AfsR (Horinouchi *et al.*, 1990), the four proteins still may represent a family of DNA-binding regulatory proteins that recognize specific nucleotide sequences.

To understand how the *dnrI* gene functions as a transcriptional activator, we overproduced and purified the DnrI protein from *Escherichia coli* and *S. peucetius* and, through DNA–protein binding experiments, discovered that DnrI binds to the promoter regions of the putative *dnrG–dpsABCD* and *dpsEF* operons and immediately upstream of the promoter of the putative *dnrDKPSQ* operon.

Results

Identification of the dnrG–dpsABCD *and* dpsEF *promoter regions*

As the phenotype of a *dnrI* mutant (Stutzmann-Engwall *et al.*, 1992; Madduri and Hutchinson, 1995b) led to the suggestion that DnrI might be a positive regulator of the *dpsABCD* and *dpsEF* genes involved in aklanonic acid biosynthesis (Grimm *et al.*, 1994) (Fig. 1A), we sought to identify the promoter regions of these putative operons. Preliminary low-resolution S1 nuclease protection experiments with Clone A (1.1 kb *Bam*H–*Sst*I *dpsE* segment),

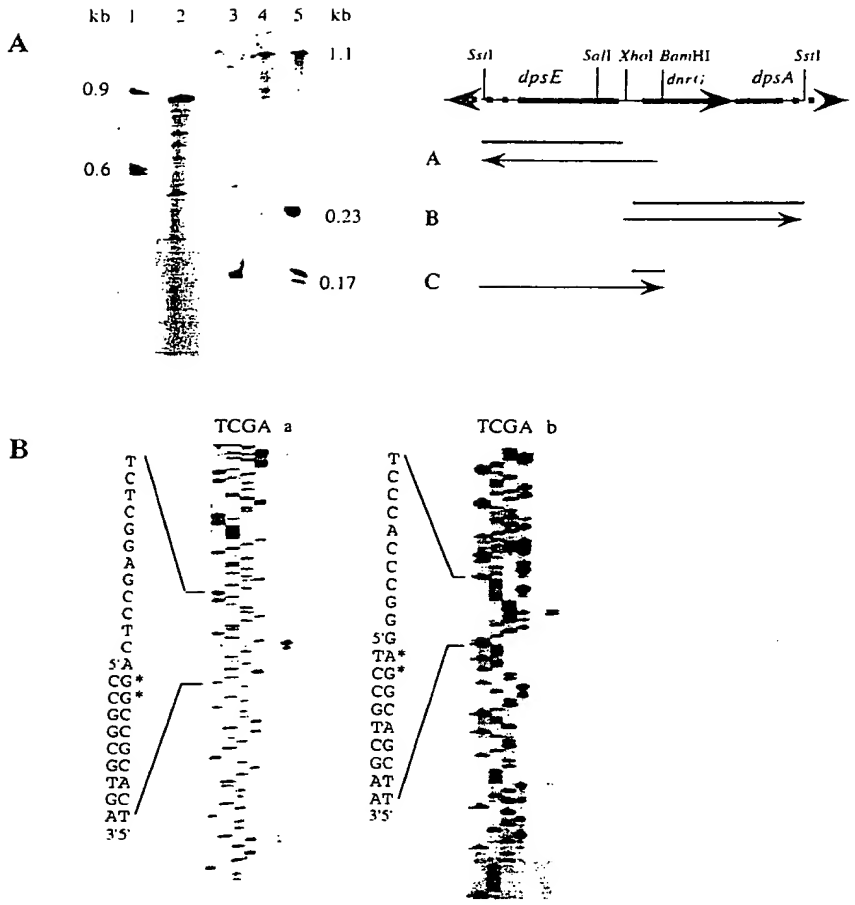

Fig. 2. A. Low-resolution S1 protection analysis of the *dpsE* and *dnrG* transcripts in *S. peucetius*. The left panel is an autoradiograph of the protected DNA fragments. Lanes: 1 and 5, digested probe fragments as molecular size markers; 2, Clone A; 3, Clone C; 4, Clone B. Clones A, B and C are the ssDNA produced from M13 clones shown in the right panel. The arrows indicate the direction of transcription. Thick lines above DNA clones A, B and C show the positions of S1 protected segments in relation to the restriction map of the intergenic region of *dpsE–dnrG*.
B. Primer-extension analysis for the *dnrG* transcript with Primer a and for the *dpsE* transcript with Primer b. TCGA are sequencing reactions generated with the corresponding primers. The asterisks indicate the apparent transcriptional start points.

Clone B (1.1 kb *Xho*I–*Sst*I *dnrG* segment) and Clone C (1.1 kb *Sst*I–*Bam*HI *dnrG* segment), produced a 0.9 kb protected fragment with A, a 0.17 kb protected fragment with C, and a 1.05 kb protected fragment with B (Fig. 2A). These results suggested that the 5′ end of the *dpsE* transcript is located shortly upstream of the predicted *dpsE* ATG start codon, and the 5′ end of the *dnrG–dpsABCD* transcript is within the region defined by clones B and C, ≈100 bp upstream of the predicted *dnrG* ATG start codon (Grimm *et al.*, 1994). Primer-extension experiments were then performed to locate the apparent transcriptional start points precisely as two adjacent bases 17 and 18 nucleotides (nt) upstream of the *dpsE* ATG start codon and 86 and 87 nt upstream of the *dnrG* ATG start codon (Fig. 2B).

Overexpression and purification of DnrI

To obtain sufficient quantities of purified DnrI for *in vitro* experiments, we overproduced the protein in *S. peucetius*. As our prior experiments had shown that overexpression of *dnrI* in an otherwise wild-type strain was inhibitory to growth (Stutzman-Engwall *et al.*, 1992), we used a strain (WMH1535) deleted for *dpsB* (Grimm *et al.*, 1994), in order to minimize synthesis of potentially deleterious intermediates of DNR biosynthesis. When *dnrI* was expressed from the strong, constitutive *ermE** (Schmitt-John and Engels, 1992; Bibb *et al.*, 1994) or thiostrepton-inducible *tipA* (Kuhstoss and Rao, 1991; Takano *et al.*, 1995) promoters on plasmids pWHM1104 (p*ermE**) and pWHM1111 (p*tipA*) (Fig. 3 and see Table 1 later) to synthesize the native DnrI protein, DNR and RHO production was restored to the *dnrN* null mutant WMH1530 (Otten *et al.*, 1995) (data not shown). This result suggests that synthesis of DnrI from a heterologous promoter can bypass the need for DnrN, which, in turn, positively regulates transcription of DNR biosynthesis genes.

Despite the apparent overproduction of DnrI from heterologous promoters in *S. peucetius*, we were unable to detect DnrI in extracts of the overproducing cells by Coomassie blue-staining of proteins subjected to SDS–PAGE. Bands corresponding to DnrI could by detected by immunoblotting in the extracts from the pWHM1111-containing strain after thiostrepton-induction, and in extracts from the

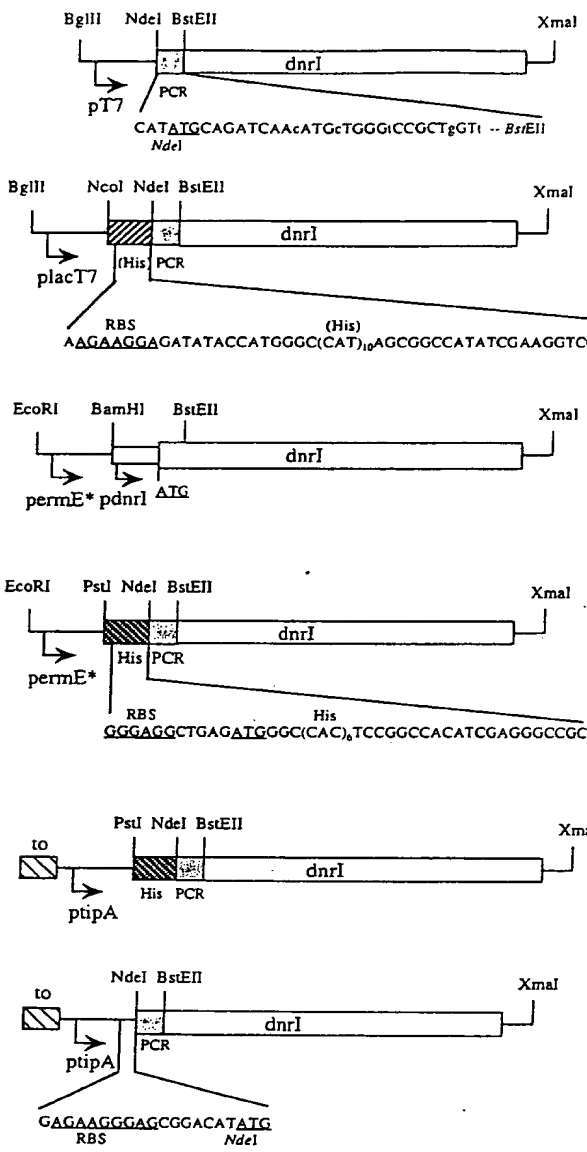

Fig. 3. Structure of expression plasmids carrying the *dnrI* gene to overproduce DnrI in *E. coli* and *S. peucetius*. The plasmids named inside the parentheses are the vectors used to construct the expression plasmids.
t_o = transcription terminator.

pWHM1104-containing strain when it was grown in GPS medium (Dekleva *et al.*, 1985) but not in R2YE medium (Fig. 4, lanes 1 to 5). The His-tagged DnrI protein (32.2 kDa) was also detected in the extract from thiostrepton-induced culture of the pWHM1110-containing strain, but not in the pWHM1108-containing strain in both the R2YE and GPS media (Fig. 4, lanes 6 to 10). These results indicate that expression of *dnrI* from the *tipA* promoter seems higher than that from the *ermE** promoter, and that *dnrI* expression from the *dnrI* promoter (Madduri and Hutchinson, 1995b) in pWHM1104 is influenced by the nutrients in the growth medium.

Starting with strain WMH1535(pWHM1111), a cell-free extract was prepared after 12 h of exposure to thiostrepton. DnrI was fractionated by the addition of ammonium sulphate to the cell-free extract at between 15 and 35% of saturation and more than 70% of the DnrI protein was recovered in the ammonium sulphate pellet. Further purification was performed as described in the *Experimental procedures* by anion-exchange (Q-sepharose, Mono Q) and hydrophobic interaction (Phenyl-Superose) chromatography. The purified protein gave a major band of 29.5 kDa on a SDS–PAGE gel, in good agreement with the value predicted from the *dnrI* gene sequence (Stutzman-Engwall *et al.*, 1992) (Fig. 5A, lane 6). DnrI had a strong tendency to aggregate and stick to the matrix,

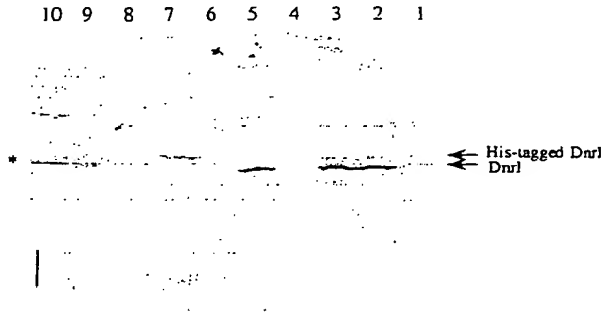

Fig. 4. Western immunoblot analysis of DnrI produced in *S. peucetius* WMH1535 transformants. Total cell lysates were subjected to SDS–PAGE (12.5%) analysis and transfered to an Immobilon-P membrane. The blot was developed with the ECL kit as described in the *Experimental procedures*. Lanes: 1–3, cell lysate of pWHM1111 with no thiostrepton-induction, with 12 h of strepton-induction, and with 36 h of thiostrepton-induction, respectively; 4, 48 h R2YE culture of pWHM1104; 5, 48 h GPS culture of pWHM1104; 6–8, pWHM1110 with no thiostrepton-induction, with 12 h of thiostrepton-induction, and with 36 h of thiostrepton-induction, respectively; 9, 48 h R2YE culture of pWHM1108; 10, 48 h GPS culture of pWHM1108. Arrows identify the position of DnrI or His-tagged DnrI protein. The bands in lanes 9 and 10 indicated by an asterisk (*) are not DnrI because they moved faster than the actual DnrI protein, which could be seen by careful comparison with the original blot.

and was eluted from columns as a very broad peak or could not be efficiently eluted, resulting in a major loss during the overall purification process. The purified DnrI protein either from *E. coli* or *S. peucetius* was very unstable and lost most of the DNA-binding activity when it was stored at −80°C in TGED buffer for one month.

As initial attempts to perform the gel mobility-shift experiments described below with protein extracts from *S. peucetius*(pWHM1104) or -(pWHM1111) transformants were successful, the *dnrI* gene was also expressed in (and DnrI purified from) *E. coli* carrying the *dnrI* gene fused to the phage pT7 late promoter. Both native DnrI and DnrI modified by a series of 6 or 10 histidine residues at its N-terminus were produced. pWHM1102 (Fig. 3) was used to produce the His-tagged DnrI protein, which was easily purified by Ni^{2+}-affinity chromatography, and pWHM1101 (Fig. 3) was used to produce native DnrI protein to develop the purification scheme described above for DnrI from *S. peucetius*. Although most of the DnrI produced was insoluble (Fig. 6), the His-tagged DnrI was purified by Ni^{2+}-affinity chromatography and yielded a single protein band of 32.2 kDa on SDS–PAGE (Fig. 6A, lane 6). Native DnrI protein (29.5 kDa) was purified to homogeneity from *E. coli* BL21(DE3)/pWHM1101 transformants by ammonium sulphate precipitation, Mono Q and Phenyl-Superose chromatography (Fig. 6B, lane 6).

DNA-binding activity of purified DnrI

To test the idea that DnrI is a direct positive regulator of *dps, dnm* and *dnr* gene expression (Madduri and Hutchinson, 1995b), we assayed the ability of purified native or His-tagged DnrI to bind to DNA fragments carrying *dps* and *dnr* promoter regions. Using gel mobility-shift assays to determine the specificity of the DNA-binding activity, we observed the progressive binding of DnrI to the intergenic regions of *dnrG–dpsE* and *dnrC–dnrD* (Fig. 7) as shown in Fig. 8. The mobility of both the end-labelled fragments F1 and F2 on native 5% PAGE was retarded by the addition of DnrI protein in the presence of poly(dI-dC):(dI-dC), and this binding was eliminated by the further addition of unlabelled DNA fragments F1 and F2 (Fig. 8, A and B, lanes 6 and 14). These data do not reflect the relative affinity of DnrI from *S. peucetius* and *E. coli* because the latter material had been stored at −80°C for one month, which can result in a significant loss of activity, presumably due to protein denaturation.

To locate the sites of interaction between DnrI in the intergenic regions of the *dnrG–dpsE* and *dnrC–dnrD* genes more precisely, DNase I footprinting analysis was conducted. When the end-labelled fragments F1* or F3 (Fig. 7A) were incubated with DNase I both in the presence and absence of DnrI, DnrI protected about 65 bp of the *dnrG–dpsE* intergenic region extending upstream from the −10 to −35 regions of the *dnrG* or *dpsE* promoters (Figs 7B, 9A and 9B). For the end-labelled fragments F2 or F4 (Fig. 7A), DnrI protected an ≈80 bp portion upstream of the *dnrD* −10 region in the *dnrC–dnrD* intergenic region (Figs 7B, 9C and 9D). For either promoter region, the region protected was judged by considering

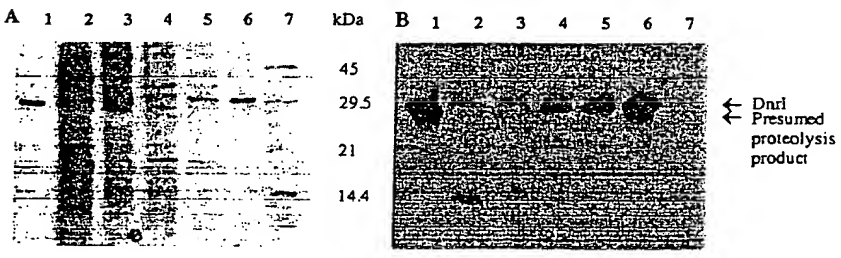

Fig. 5. Purification of the DnrI protein from *S. peucetius* WMH1535(pWHM1111).
A. 15% SDS–PAGE analysis.
B. Western immunoblot analysis with anti-DnrI antibody.
Lanes: 1, DnrI protein purified from *E. coli*(pWHM1101) as a positive control; 2, cell-free extract; 3, 15–35% ammonium sulphate precipitate; 4, after Q-Sepharose chromatography; 5, after Mono-Q chromatography; 6, after Phenyl-Superose chromatography; 7, molecular weight markers.

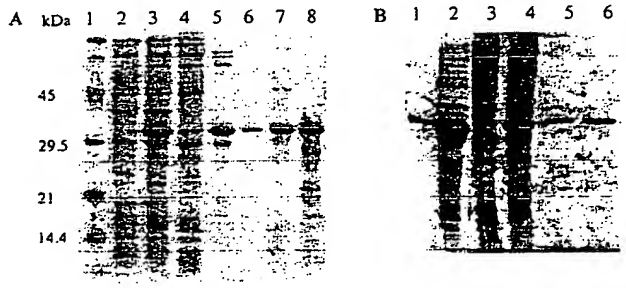

Fig. 6. Overexpression of the *dnrI* gene in *E. coli*.
A. SDS–PAGE (12.5%) analysis of His-tagged DnrI protein purified from *E. coli* BL21(DE3) containing pWHM1102. Lanes: 1, molecular weight markers; 2, total cell lysate of *E. coli* BL21(DE3)(pET16b) as a control; 3, total cell lysate of *E. coli* BL21(DE3)(pWHM1102); 4, cell-free extract (soluble fraction); 5, after the first Ni^{2+}-affinity chromatography (soluble fraction); 6, after the second Ni^{2+}-affinity chromatography (soluble fraction); 7, 6 M urea extract from cell lysate pellet (insoluble fraction); 8, after Ni^{2+}-affinity chromatography (insoluble fraction).
B. SDS–PAGE (15%) analysis of DnrI protein purified from *E. coli* BL21(DE3) containing pWHM1101. Lanes: 1, purified His-tagged DnrI as a control; 2, total cell lysate of *E. coli* BL21(DE3)(pWHM1101); 3, cell-free extract (soluble fraction); 4, 35% ammonium sulphate precipitate; 5, after Mono-Q chromatography; 6, after Phenyl-Superose chromatography.

the results for both DNA strands (Fig. 9). When the bands in lanes 2 or 3 were much fainter than expected, relative to the intensity of the bands in lanes 1, the presence of the same bands in all three lanes was taken as evidence for lack of protection. There was no significant difference in the results when these experiments were repeated with the native or His-tagged DnrI purified from *E. coli* or *S. peucetius* (data not shown). Although the data in Fig. 9, A and B suggest that DnrI protected a 65 bp region in the *dnrG–dpsE* intergenic region, which appears to be associated with the −35 regions of the two divergent promoters (Fig. 7B), the lack of mapping data for the 5′ end of the *dnrC* promoter prevents a similar distinction for the *dnrC* promoter (Fig. 7B).

Discussion

The *dnrI* gene, encoding a pathway-specific transcriptional factor, is essential for DNR biosynthesis in *S. peucetius* (Stutzman-Engwall *et al.*, 1992; Madduri and Hutchinson, 1995b). As a first step towards a better understanding of the regulatory mechanism, we have studied how DnrI interacts with three promoters of DNR biosynthesis genes.

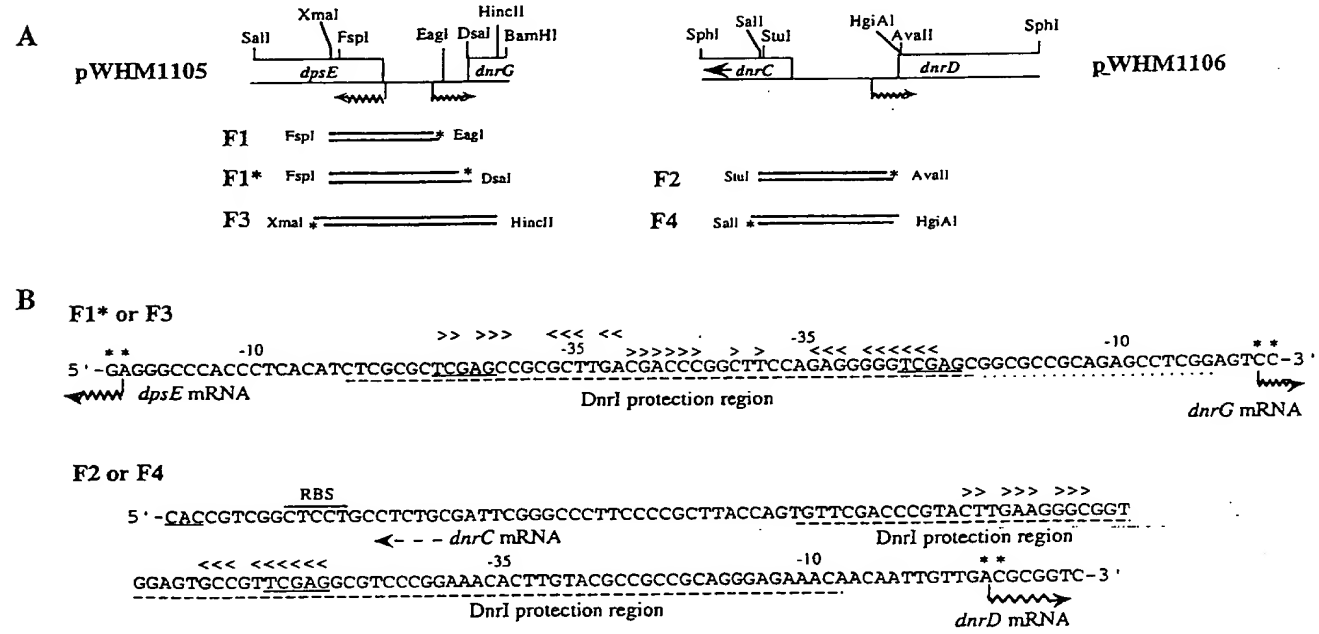

Fig. 7. A. Genomic organization of the intergenic regions of *dnrG–dpsE* (pWHM1105) and *dnrC–dnrD* (pWHM1106). F1, F1*, F2, F3 and F4 indicate the locations of the end-labelled DNA fragments used in gel mobility-shift and DNase I footprinting assays. The asterisks indicate the [α-^{32}P]-dCTP-labelled end of the DNA segments and the arrows show the apparent transcriptional start points. (B) The DNA sequences of the intergenic regions of *dnrG–dpsE* and *dnrC–dnrD*. The −35 and −10 regions of the promoter along with the corresponding apparent transcriptional start points (*) are shown as determined in the text and by Madduri and Hutchinson (1995a). The regions protected from DNase I digestion by purified DnrI protein are indicated by broken lines. The dotted line indicates the DnrI partial protection region. The conserved sequence 5′-TCGAG-3′ in the protected region is underlined. Imperfect inverted repeats are indicated by arrowheads above the sequence. The putative ribosome-binding site of *dnrC* is overlined and its predicted translational start codon is underlined.

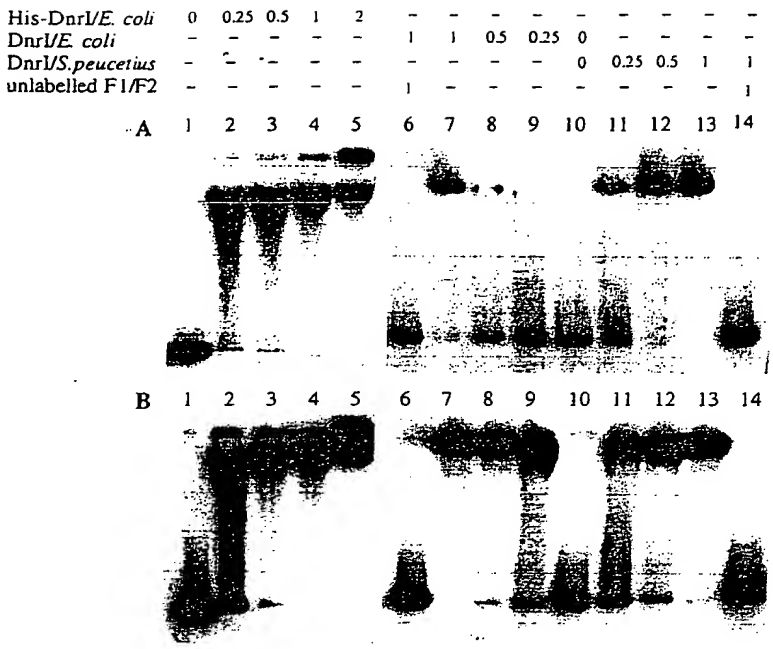

Fig. 8. Gel mobility-shift analysis of [α-^{32}P] end-labelled DNA fragments F1 (A) and F2 (B) with purified native or His-tagged DnrI protein in the presence of 1.5 μg of poly(dC-dI):(dC-dI). Lanes: 1 and 10, no protein; 2–5, His-tagged DnrI; 6–9, DnrI purified from *E. coli*; 11–14, DnrI purified from *S. peucetius*; 2, 9, and 11, 0.25 μg of protein; 3, 8, and 12, 0.5 μg of protein; 4, 7, and 13, 1 μg of protein; 5, 2 μg of protein; 6 and 14, 1 μg of the specific competitor: unlabelled DNA fragments F1 (A) and F2 (B).

The DnrI protein was isolated from both *E. coli* and *S. peucetius* to allow assessment of whether its properties were host dependent, for instance to determine if its activity depends on some type of post-translational modification. Attempts to overexpress the *dnrI* gene in *S. peucetius* WMH1535 using a high-copy-number vector with the strong, constitutively expressed *ermE** promoter led to a very low level of DnrI relative to that produced using the thiostrepton-inducible *tipA* promoter. As induction of the *tipA* promoter requires the TipA protein *in vivo* (Takano *et al.*, 1995), a homologous *tipA* gene must also be present in *S. peucetius*. The amount of DnrI overproduced and purified from *S. peucetius* was much less than that obtained from *E. coli*, partly because of an apparent toxicity that was ameliorated by using the DNR nonproducing *dpsB* mutant. Regardless of this, the DnrI purified from both hosts was extremely unstable, undergoing rapid aggregation and precipitation resulting in a low yield of the purified soluble protein.

As the DnrI protein purified from *S. peucetius* showed behaviour on the chromatography columns and DNA-binding activity identical to those from *E. coli*, it is unlikely that this protein undergoes post-translational modification such as phosphorylation prior to the activation of gene transcription in *S. peucetius*. Moreover, purified DnrI was not phosphorylated by [^{32}P]-acetyl phosphate under *in vitro* conditions that led to the detectable labelling of purified PhoB due to its phosphorylation (K. Furuya *et al.* unpublished).

Although we examined the binding of DnrI to only three promoter regions in this work, *dnrG*, *dpsE* and *dnrD*, which control the expression of genes acting at early (*dnrG–dpsABCD* and *dpsEF*) and late (*dnrDKPQS*) stages in DNR biosynthesis (Fig. 1), it is likely that DnrI binds to other *dnr* gene promoters. The binding sites of most transcriptional regulators (activators or repressors) either overlap the RNA polymerase-binding site or are located immediately upstream of this sequence (Collado-Vides *et al.*, 1991). This is also true for DnrI because its binding sites overlap the promoter sequences it activates (Fig. 7B). Each of the three regions protected by DnrI from DNase I digestion contains an imperfect inverted repeat and the sequence 5′-TCGAG-3′. Although both of these motifs might serve as DnrI recognition sequences, this idea remains speculative until tested by site-specific mutation experiments. (Comparison of the DNA sequences covering about 200 bp upstream of the predicted translational start codons for all other putative operons or genes in the cluster of DNR biosynthesis genes (Fig. 1B) revealed that the sequence 5′-TCGAG-3′ is present in only 4 out of 10 possible or actual promoter regions.) Consequently, we believe that *dnrI* controls many of the DNR biosynthesis genes coding for early- and late-acting enzymes by binding near to the −35 region in the promoters of the target genes to effect transcriptional activation. Moreover, as *dnrI* suppresses the effect of an *actII–ORF4* mutation (Stutzman-Engwall *et al.*, 1992) to restore the blue pigmentation characteristic of ACT, the ActII–Orf4 protein also may recognize a specific DNA sequence in promoters of ACT biosynthesis genes.

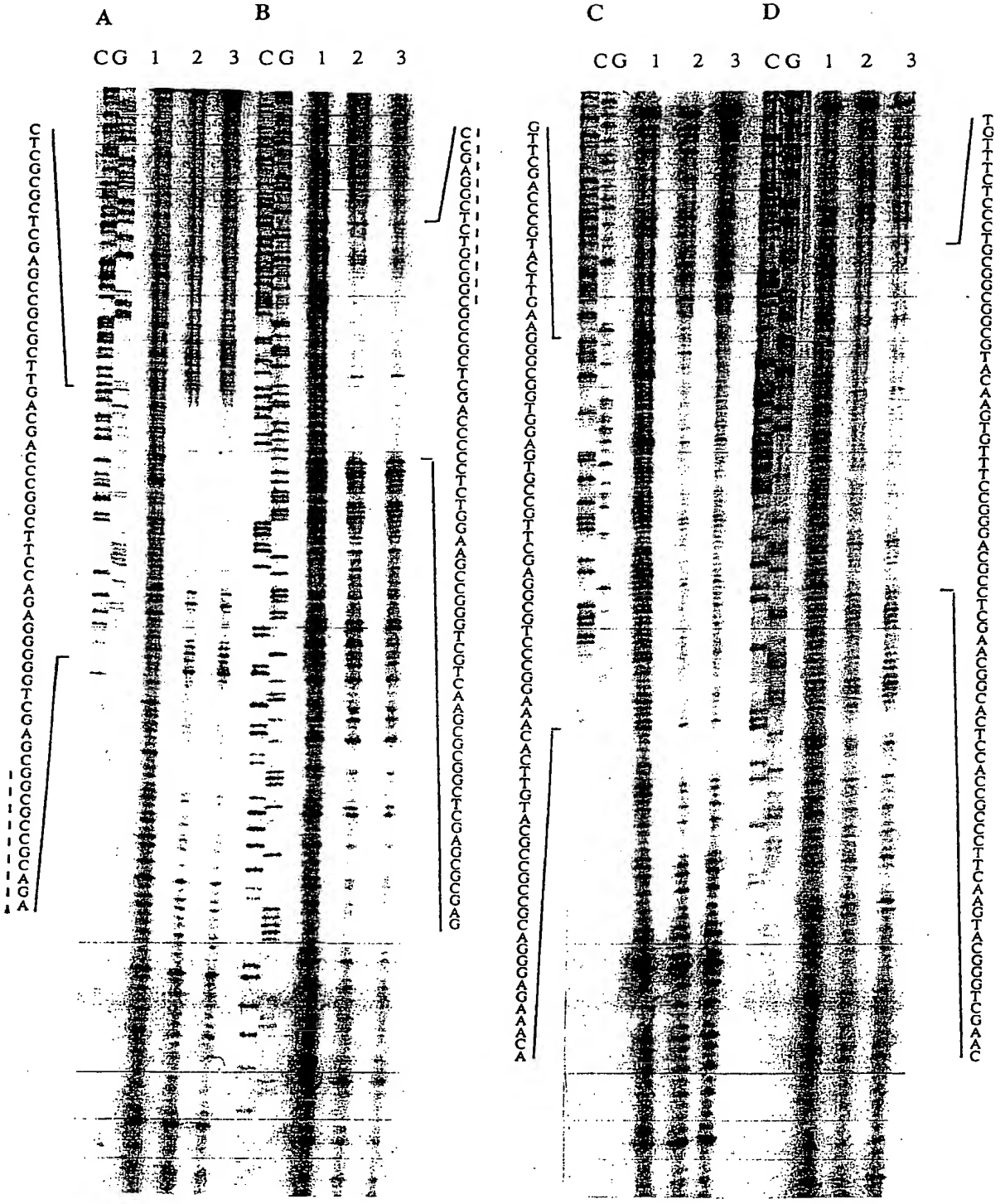

Fig. 9. DNase I footprinting analysis to identify the DnrI-binding sites. DnrI, purified from *E. coli*(pWHM1101) as described in the *Experimental procedures*, protected portions of the intergenic regions of the *dnrG–dpsE* (panel A for the upper strand, using the F1* fragment; panel B for the lower strand, using the F3 fragment) and *dnrC–dnrD* genes (panel C for the upper strand, using the F2 fragment; panel D for the lower strand, using the F4 fragment). Lanes: 1, no protein; 2, 1 μg of protein; 3, 2 μg of protein. Lanes labelled CG are sequencing reactions generated with the corresponding primers described in the *Experimental procedures*. The broken lines in (A) and (B) indicate the region of DnrI partial protection.

Experimental procedures

Bacterial strains and plasmids

E. coli DH5α (Sambrook *et al.*, 1989) and BL21(DE3) (Novagen) were used for subcloning and expression hosts, respectively. The pT7-7 (Tabor, 1990) and pET16b (Novagen) plasmids were used as the expression vectors in *E. coli* BL21(DE3). Plasmids containing the *ermE** promoter (pWHM879) and the *ermE**–His-tagged cassette (pUR11) were obtained from G. Meurer (G. Meurer and C. R. Hutchinson, unpublished) and U. Roos (U. Roos and C. R. Hutchinson, unpublished), respectively. Plasmids pWHM75 and pWHM920 with the intergenic regions of the *dnrG–dpsABCD* and *dpsEF* genes, and the *dnrC* and *dnrDKPQS* genes, were obtained from A. Grimm (Grimm *et al.*, 1994) and K. Madduri (Madduri and Hutchinson, 1995a), respectively. Other plasmids and strains used in this study are listed in Table 1.

Media and growth conditions

E. coli strains were grown in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) at 37°C and transformants were selected with 100 μg ml^{-1} ampicillin or 50 μg ml^{-1} kanamycin. *S. peucetius* strains were grown at 30°C on ISP4 medium (Difco Labs) for spore preparation and in R2YE medium (Hopwood *et al.*, 1985) for preparation of protoplasts and transformations as described previously (Tang *et al.*, 1994). Plasmid-containing *Streptomyces* strains were selected with 25 μg ml^{-1} thiostrepton or 10 μg ml^{-1} kanamycin. Anthracycline production was determined by high-performance liquid chromatography (HPLC) analysis of culture extracts from the GPS complex production medium (Dekleva *et al.*, 1985) as described by Otten *et al.* (1995).

DNA manipulation

Plasmid DNA isolation and transformation were carried out as described previously (Tang *et al.*, 1994). Oligonucleotide primers were synthesized on a polymerase chain reaction (PCR) DNA synthesizer (Model 391, Applied Biosystems) and purified on an 8 M urea/16% polyacrylamide gel by electroelution from gel slices. The PCR was carried out with a DNA thermocycler Model 480 (Perkin Elmer Cetus). PCR mixtures consisted of 30 ng of pWHM358 (1 μl) as a template, 0.5 μg (1 μl) each of Primer 1 (5′-GGCATATGCAGATCAACATGCTGGGTCCGCTGGTTCCA-3′) and Primer 2 (5′-GTGGATCCTTGTCGGGGCGCGGTCAGGC-3′) in 20 mM Tris-HCl, pH 8.3, 1.2 mM $MgCl_2$, 20 mM KCl, 0.1% Triton X-100, 100 mg BSA, 5% formamide, 50 mM dNTP and 4.5 U of Taq polymerase in a final volume of 100 μl. The cycling conditions were 50 s of denaturation at 96°C and 1.5 min of annealing/extension at 70°C. The PCR products were purified by agarose gel with a QIAEX kit (QIAGEN Inc.).

To prepare radiolabelled DNA fragments for DNA-binding assays, a 0.49 kb *SalI–Bam*HI fragment from pWHM75, containing the divergent promoter regions for the *dnrG–dpsABCD* and *dpsEF* genes, and a 0.64 kb *Sph*I fragment from pWHM920, containing the divergent promoter regions for the *dnrC* and *dnrDKPQS* genes, were subcloned into similar sites of pUC18 (Yanisch-Perron *et al.*, 1985) to yield pWHM1105 and pWHM1106, respectively (Fig. 7A). A 205 bp *EagI–Fsp*I fragment (F1) of pWHM1105, a 255 bp *DsaI–Fsp*I fragment (F1*) of pWHM1105, a 340 bp *XmaI–Hin*cII fragment (F3) of pWHM1105, a 244 bp *AvaII–StuI* fragment (F2) of pWHM1106 and a 245 bp *SalI–HgiA*I fragment of pWHM1106 (Fig. 7A) were end-labelled with [α-^{32}P]-dCTP (Amersham) and Klenow polymerase. The labelled probes were separated from unincorporated [α-^{32}P]-dCTP by repeated fitration through an ultrafree CL filter (Millipore Corp.).

Transcriptional analysis of the putative dnrG–dpsABCD *and* dpsEF *operons*

Total RNA was isolated as described by Guilfoile and

Table 1. Selected plasmid strains used in this work.

Plasmid/Strain	Description	References
Plasmid		
pWHM1101	The *dnrI* gene under the control of the T7 RNA polymerase promoter in pT7-7	This work
pWHM1102	A N-terminal His-tagged *dnrI* gene under control of the *T7lac* promoter in pET16b	This work
pWHM1104	The *dnrI* gene under control of the tandem *ermE** and *dnrI* promoters in pWHM3	This work
pWHM1105	The intergenic regions of *dpsE* and *dpsABCD* on pUC18	This work
pWHM1106	The intergenic regions of *dnrC* and *dnrDKPQS* on pUC18	This work
pWHM1108	A N-terminal His-tagged *dnrI* gene under the control of the *ermE** promoter in pWHM3	This work
pWHM1109	A high-copy *E. coli* and *Streptomyces* shuttle vector carrying the *tipA* promoter derived from pWHM3 and pIJ6021	This work
pWHM1110	A N-terminal His-tagged *dnrI* gene under the control of the *tipA* promoter in pWHM1109	This work
pWHM1111	The *dnrI* gene under the control of the *tipA* promoter in pWHM1109	This work
pWHM358	The *dnrIJ* genes in pGEM7zf(+)	Stutzman-Engwall *et al.* (1992)
pWHM3	A high-copy *E. coli* and *Streptomyces* shuttle vector based on pIJ702 and pUC19	Vara *et al.* (1989)
pIJ6021	A high-copy *Streptomyces* vector carrying the *tipA* promoter based on pIJ486	Takano *et al.* (1995)
Strain		
WMH1535	*dpsB*-deleted mutant of *S. peucetius* ATCC 29050	Grimm *et al.* (1994)
WMH1530	*dnrN*::*aphII*-disrupted mutant of *S. peucetius* ATCC 29050	Otten *et al.* (1995)

Hutchinson (1992) from 72 h APM cultures (Guilfoile and Hutchinson, 1991) of *S. peucetius* ATCC 29050. Low-resolution S1 mapping experiments were performed using M13 clones containing single-strand (ss) DNA complementary to the mRNA for hybridization and S1 digestion (Tang and Hutchinson, 1993). Primer-extension analysis was conducted by a modification of the method of Stein *et al.* (1989) with two 30-mer oligodeoxynucleotide primers: (a), 5′-GCTGT-GGCATCGCTGCTCCACGGGTCCGTT-3′, beginning 10 bp downstream of the predicted *dnrG* ATG start codon and complementary to the *dnrG–dpsABCD* polycistronic mRNA; and (b), 5′-GAACACCCGGGTCCCGTCCTGGGCGAGCTT-3′, beginning 102 bp downstream of the predicted *dpsE* ATG start codon and complementary to the *dpsEF* mRNA. The resulting primer-extension products were analysed on a sequencing gel along with dideoxy DNA sequencing ladders made with the same primers.

Construction of dnrI *expression plasmids*

To prepare pWHM1104, a 1.2 kb *Pst*I–*Xma*I fragment of pWHM358 (Stutzman-Engwall *et al.*, 1992) that contains the promoter and entire coding region of the *dnrI* gene was cloned into the same sites of pWHM879 downstream of the *ermE** promoter. The resulting plasmid was digested with *Eco*RI–*Nsi*I, and a 1.6 kb fragment (p*ermE**::*dnrI*) was cloned into *Pst*I–*Eco*RI sites of pWHM3 (Vara *et al.*, 1989) to give pWHM1104 (Fig. 3).

To construct the other expression plasmids, the PCR was used with site-specifically modified oligodeoxynucleotides to introduce a *Nde*I site at the predicted *dnrI* translational start codon (Stutzman-Engwall *et al.*, 1992) as described above. The final PCR product was filled in with Klenow polymerase and ligated into the *Hin*cII site of pUC18. The resulting plasmid was digested with *Bst*EII–*Xma*I to remove most of the *dnrI* gene synthesized by the PCR and replaced with the 0.9 kb *Bst*EII–*Xma*I fragment of pWHM1104 to give pWHM1100, whose 57 bp *Nde*I– *Bst*EII PCR segment was verified by DNA sequence analysis.

The 1.0 kb *Nde*I–*Xma*I fragment from pWHM1100 containing the *dnrI* gene was cloned into the same sites of pT7-7 to give pWHM1101. The entire *dnrI* gene was transferred from pWHM1101 as an 1.0 kb *Nde*I–*Bam*HI fragment into similar sites of pET16b (Novagen), pWHM1109 and pUR11 to yield pWHM1102, pWHM1111 and pWHM1112, respectively. *E. coli–Streptomyces* shuttle vector pWHM1109 was prepared by ligation of a 3.3 kb *Xho*I–*Eco*RI fragment from pWHM3 and a 6.0 kb *Xho*I–*Eco*RI fragment from the *tipA* promoter containing plasmid pIJ6021 (Takano *et al.*, 1995). The 1.05 kb *Pst*I–*Bam*HI and 1.45 kb *Eco*RI fragments of pWHM1112 were transferred into the same sites of pWHM1109 and pWHM3 to yield pWHM1110 and pWHM1108, respectively (Fig. 3).

Protein analysis

Protein concentrations were determined according to the Bradford method (Bradford, 1976), with BSA as the standand. SDS–PAGE was performed according to the method of Laemmli (1970) or on the Phastsystem (Pharmacia Biotech Inc.) as described by the manufacturer, and the gels were stained with Coomassie blue R. Western immunoblotting was performed using a Bio-Rad electroblotting apparatus, and proteins were transferred to polyvinylidine difluoride (Immobilon-P; Millipore) membranes. The immunodetection assay was processed with an ECL kit as instructed by the manufacturer's protocol (Amersham Life Science). A goat anti-rabbit IgG horseradish peroxidase conjugate in a 1:3000 dilution was used as the secondary antibody.

Preparation of anti-DnrI protein antibody

Purified insoluble His-tagged DnrI protein (1 mg, see below) in TBS buffer (20 mM Tris-HCl, pH 7.6 in 0.9% NaCl (w/v)) was mixed with Freund's complete adjuvant and injected into two rabbits (6 lb, Hazelton) by the intradermal route at about 30 sites. The rabbits were booster-injected every four weeks with a further 1 mg of His-tagged DnrI protein and were bled 2 weeks after each booster injection. The antiserum was brought to between 35% and then 45% of saturation with solid ammonim sulphate to precipitate the antibody. The anti-DnrI antibody was dialysed against TBS buffer and stored at −80°C.

Purification of the DnrI protein

Throughout the purification procedure, the DnrI protein was identified by SDS–PAGE and Western immunoblot analysis with the anti-DnrI antibody. All of the operations were performed at 4°C, except for the fast-protein liquid chromatography (FPLC), which was carried out at room temperature. The FPLC system and chromatography columns were purchased from Pharmacia Biotech Inc.

His-tagged DnrI protein from E. coli. *E. coli* Bl21(DE3) harbouring plasmid pWHM1102 was grown in LB medium at 37°C to an optical density (OD_{600}) of 0.6, and IPTG was added to a final concentration of 1 mM. After incubation for 3.5 h at 28°C, cells were harvested, washed with binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) and disrupted by sonication. The cell lysate was centrifuged at 15 000 r.p.m. for 15 min at 4°C and the resulting supernatant containing soluble DnrI was loaded onto a Ni^{2+}-chelating column (2 ml bed) as directed by the manufacturer (Novagen). Soluble His-tagged DnrI protein was eluted with 1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, and dialysed against TGED buffer (10 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol, 1 mM EDTA, 1 mM DTT) overnight. The final protein samples were divided into 100 μl aliquots and stored at −80°C for DNA-binding assays. The insoluble pellet from the cell lysate, which contained most of the DnrI protein, was resuspended in the binding buffer with 6 M urea and incubated on ice for 1 h. The remaining insoluble material was removed by centrifugation as above and the supernatant was loaded onto a denaturing Ni^{2+} column (the buffers contained 6 M urea) as directed by the manufacturer (Novagen). The denatured His-tagged DnrI protein was eluted with 100 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9 and dialysed against TBS buffer to remove the urea, upon which the purified DnrI protein reprecipitated.

DnrI protein from S. peucetius. *(i) Preparation of crude extract.* Cultures of *S. peucetius* WMH1535 Δ*dpsB* (Grimm *et al.*, 1994) containing pWHM1111 were grown in R2YE medium with 10 μg ml^{-1} kanamycin in a 2 l baffled flask at 30°C and 300 r.p.m. for 12 h, then thiostrepton was added to a final concentration of 10 μg ml^{-1}. After a further 12 h of incubation, the mycelial cells were harvested, washed and resuspended with Buffer B (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM DTT, 0.01% NP-40 (v/v)) plus 0.2 mM phenylmethysulfonyl fluoride. Cells were disrupted by sonication and cell debris was removed by centrifugation to yield a cell-free extract. Proteins in the cell-free extract were precipitated with 15–36% saturated ammonium sulphate and collected by centrifugation.

DnrI protein from S. peucetius. *(ii) Q-Sepharose anion-exchange column.* As DnrI constituted only a small fraction of the total protein, the Q-Sepharose column was used to remove most of the contaminating proteins that bound to Q-Sepharose equilibrated with Buffer B. The 15–36% ammonium sulphate pellet was dissolved in Buffer B and dialysed against 5 l of the same buffer overnight with three changes. After removal of the precipitated sample by centrifugation, the soluble proteins were applied to a Q-Sepharose fast-flow column (1.6 × 30 cm) that had been equilibrated with Buffer B. The pass-through fraction was collected and dialysed against buffer C (20 mM diethanolamine (DEA), pH 8.8, 0.2 mM EDTA, 0.2 mM DTT, 0.01% NP-40 (v/v)) overnight and applied to a Q-Sepharose column which had been equilibrated with Buffer C. After being washed with the same buffer, proteins were eluted with 150 ml of Buffer C containing 100 mM NaCl and were then concentrated by ultrafiltration through a filter (10 kDa cut-off; Amicon) and dialysed against Buffer C.

DnrI protein from S. peucetius. *(iii) Mono Q column.* The protein sample from the Q-Sepharose column was applied to a Mono-Q FPLC column (HR10/10) and eluted with a linear gradient from 0 to 0.5 M NaCl in Buffer C and 5 ml fractions were collected.

DnrI protein from S. peucetius. *(iv) Phenyl-Superose column.* The fractions containing DnrI protein from the Mono-Q column were brought to 15% saturated ammonium sulphate and applied to a Phenyl-Superose (HR5/5) hydrophobic interaction column. The proteins were eluted with a linear gradient from 1.2 to 0 M ammonium sulphate and 0 to 15% ethylene glycol (v/v) in Buffer D (10 mM DEA pH 8.4, 0.2 mM EDTA, 0.2 mM DTT, 0.01% NP-40) and 2 ml fractions were collected. The purified DnrI protein was dialysed against TGED buffer and stored at −80°C.

Native NdrI protein from E. coli. The soluble DnrI protein from *E. coli* BL21(DE3)/pWHM1101 transformants was recovered from the cell-free extract of a 0.4 mM IPTG induction culture by ammonium sulphate precipitation, Mono-Q and Phenyl-Superose chromatography, which was carried out as described above but without the Q-Sepharose chromatography step. The DnrI purified from *E. coli* and *S. peucetius* behaved identically on the chromatography columns.

DNA–protein binding assays

The gel mobility-shift assays were performed essentially as described by Vujaklija *et al.* (1993). End-labelled DNA fragments (5000 to 20 000 c.p.m.) were incubated with 1.5 μg of poly(dI-dC):(dI-dC) and purified DnrI protein as described in the *Experimental procedures* in a 20–30 μl total volume of TGED buffer plus 50 mM KCl at room temperature for 15 min. Protein-bound and free DNA were resolved on 5% non-denaturing polyacrylamide gels run in a high-ionic-strength buffer containing 50 mM Tris, 380 mM glycine and 2 mM EDTA, pH 8.5. The gels were dried and exposed to Kodak X-ray film.

DNase I footprinting assays were carried out with 30 μl of the DNA-binding reaction mixture described above. After incubation at room temperature for 15 min, 3 μl of DNase I solution (5 U ml^{-1} DNase I (Boehringer Mannheim) in 100 mM $MgCl_2$, 100 mM DTT) was added to each reaction mixture and incubated for 40 s at 37°C. The DNase I digestions were stopped by the addition of 7.5 μl of DNase I stop solution (3 M ammonium acetate, 0.25 M EDTA, pH 8.0, and 0.1 mg ml^{-1} tRNA) and then the samples were precipitated with ethanol. The material obtained from the DNase-I-only samples gave a compact pellet, but the material from the DNase I + DnrI samples gave a rather diffuse pellet. This may have resulted in the partial loss of precipitated nucleic acids, which could be one reason why the bands were fainter than expected in some lanes in Fig. 8. The resulting pellet was resuspended in 5 μl of TE buffer (Sambrook *et al.*, 1989) and 3.5 μl of sequencing loading buffer (US Biochemicals) and applied to a 6% polyacrylamide/13% formamide/8 M urea sequencing gel along with dideoxy DNA sequencing ladders made with Primer 3 (5′-CACGGGTCCGTTGGTCCA-3′) for Fragment F1*, Primer 4 (5′-GACCACATCGATCTGCG-3′) for Fragment F2, Primer 5 (5′-CCGGGTCCCGTCCTG-GGC-3′) for Fragment F3, and Primer 6 (5′-TCGACGACTG-GTCGAAGG-3′) for Fragment F4. After electrophoresis, the gels were dried and analysed by autoradiography.

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To Kristina
with best wishes
David

Genetic Contributions to Understanding Polyketide Synthases

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Studies on a Second and Third Ring Cyclization in Anthracycline Biosynthesis

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This paper focuses on study of second and third ring cyclization in anthracycline biosynthesis by a heterologous gene expression. Firstly, anthracycline non-producing *Streptomyces peucetius* mutant, D2 was heterologously complemented to produce daunomycins with plasmids pSgs44 and pSYE66, which contain putative cyclase genes of *S. galilaeus* and *S. nogalater*, respectively. A point mutation in the cyclase gene *dpsY* of D2 has changed glycine to serine resulting inactivation of the enzyme. Secondly, the putative cyclase gene *snoaM* from *S. nogalater*, was expressed in a gene cassette in *S. lividans* TK24 and *S. coelicolor* CH999 to study the influence of the cyclase gene on auramycinone production and the impact of endogenous genes on production profiles. The results obtained confirms that a cyclase closing the second and third ring of a polyketide is essential in anthracycline biosynthesis.

Daunomycin[1] (see Fig. 1 for structure) and especially its 14-hydroxyl derivative, doxorubicin[2], are the most widely used cytotoxic antibiotics in cancer chemotherapy. After their discovery, the biosynthesis of daunomycins and other anthracyclines has been studied intensively[3–10]. All known anthracyclines produced by streptomycetes are generated *via* a similar polyketide pathway. The enzyme complex responsible for biosynthesis of anthracycline polyketide moiety is type II polyketide synthase (PKS II). The diversity of these aromatic polyketide antibiotics arises from structural changes in the aglycone and/or in the sugars attached to the aglycone. The first stable intermediate consists of a 21-carbon aglycone skeleton, and is called aklanonic acid, AA[11]. The earlier biosynthesis intermediates before AA are unstable by their chemical nature or due to the used isolation methods and thus are usually detected as shunt products.

Although the polyketide steps leading to the aglycone are well-studied, there has been speculation whether the second and third ring closures are spontaneous, or whether they require a specific enzyme or enzymes to occur. As the PKS genes are similar in different *Streptomyces* species, it is possible to study gene functions by heterologous gene expression. Here, we report the characterization of D2, a *S. peucetius* var. *caesius* mutant, which produces shunt products derived from unstable biosynthetic intermediates. Originally, D2 was complemented with plasmids expressing putative cyclase genes from *S. galilaeus* and *S. nogalater* leading to restoration of daunomycins production. Later, by introducing a putative cyclase gene isolated from the wild type in D2, the production was restored. Furthermore, the putative cyclase gene of *S. nogalater*, *snoaM*, was expressed in *S. lividans* TK24 and *S. coelicolor* CH999 along with nine other biosynthesis genes to further clarify the role of this enzyme in anthracycline biosynthesis and the impact of endogenous genes on production profiles.

Materials and Methods

Bacterial Strains

Streptomyces peucetius var. *caesius* ATCC 27952 was

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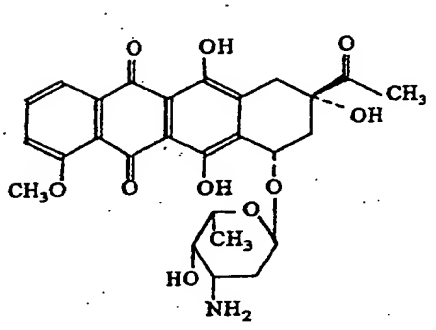
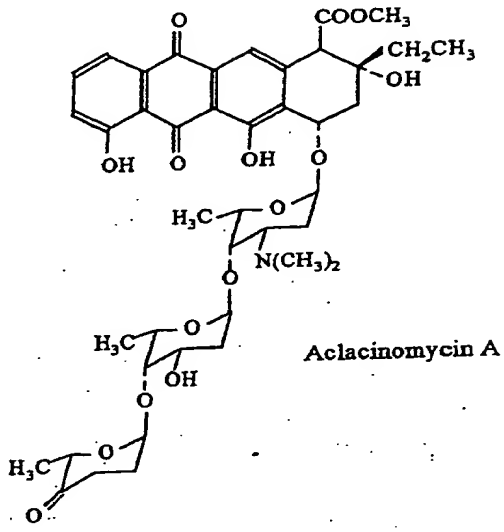
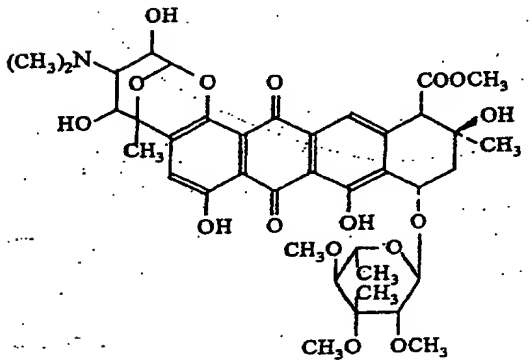
Fig. 1. Structures of nogalamycin, aclacinomycin A, daunomycin and actinorhodin.



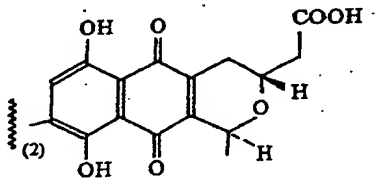
Daunomycin



Aclacinomycin A



Nogalamycin



Actinorhodin

used for mutagenesis. As host strains, *S. lividans* TK24[12] and *S. coelicolor* CH999[13] were used. The plasmids to be introduced into *S. peucetius* strains were propagated in TK24 in order to improve the transformation efficiency. Anthracycline biosynthesis genes were cloned from *S. nogalater* ATCC 27451 and *S. galilaeus* ATCC 31615. The bacterial strains used are listed in Table 1.

Mutagenesis and Mutant Selection

Cultures grown for NTG mutagenesis were incubated in 250 ml Erlenmeyer flasks containing 50 ml of Tryptone Soya Broth (TSB, Oxoid) and a spring to disperse the mycelium during aeration. All cultivations were performed in an incubator shaker (30°C, 330 rpm), unless otherwise stated. Mycelia for NTG mutagenesis were inoculated from 2-day parental culture broth (1:50, by volume) and cultivated for one day. pH of the culture was adjusted to 8.5 with 2% NaOH, and the culture was divided into two parts. One part was used as a control, whereas the other half was treated with 800 μg NTG ml^{-1} for 20 minutes at 37°C in a shaker. The NTG-treated and control cultures were then centrifuged to remove supernatant, and the cells were resuspended in 50 ml of TSB medium. Cells were grown overnight (30°C, 330 rpm), and serial dilutions (10^{-1}~10^{-6}) of the culture were made in TSB medium. The dilutions were plated on ISP4 plates (Difco) to determine the killing frequency. R2YE plates supplemented with 50 μg/ml spectinomycin[12] were used to detect mutations occurring in treated mycelia. The mutation frequency was estimated from the number of spectinomycin resistant colonies and was 10^{-3}~10^{-4}%, while the killing frequencies were over 90%. The NTG treated culture was diluted and plated on ISP4 agar plates to select colonies differing from the wild type in color or in the ability to form spores. Finally, the selected colonies were picked up, cultured and studied for anthracycline production.

General DNA Manipulations

DNA propagated in *E. coli* was ligated into pIJ486 derivatives, and introduced into *S. lividans* TK24.

Table 1. Bacterial strains and plasmids used.

Strains and plasmids	Characteristics	Reference or source
S. peucetius var. *caesius* ATCC 27952	Daunomycin producer	2
S. lividans TK24	Cloning host	12
S. coelicolor CH999	Cloning host	13
S. nogalater ATCC 27451	Nogalamycin producer	34
S. galilaeus ATCC 31615	Aclacinomycin A producer	27
D2	Anthracyclines non-producing mutant of *S. peucetius*	This work
pIJ486	*Streptomyces* plasmid	35
pIJE486	*ermE* promoter	36
	cloned into polylinker of pIJ486	8
pSY21	*snoa1-3, snorA* in pIJ486	8
pSY15	*snoa1-3, snorA, snoaB-E* in pIJ486	14
pSY15b	*snoa1-3, snorA, snoaB-E, aknH, dauE* in pIJ486	10
pSY42	*snoaL, gK, gC, gG2, gN, aM, gA, gJ* in pIJ486	16
pSYE66	*snoaM* in pIJE486	This work
pSgs4	*aknS, T, U, V, W, X2, Y* in pIJE486	18
pSgs44	*aknW, X2, Y* in pIJE486	18
pMC9	*snoa1-3, snoaB-E, aknH, dauE* in pIJE486	Unpublished
pMC10aM	*snoaM* added to pMC9	This work
pDpsY	*S. peucetius* var. *caesius* ATCC 27952 *dpsY* in pIJE486	This work

Subsequently, plasmid DNA isolated from TK24 was introduced into *S. peucetius*. All *Streptomyces* strains were transformed by standard methods[12] with minor modifications[14]. DNA isolation and manipulation were carried out by standard procedures[12,15].

Three putative polyketide cyclase genes were amplified by PCR using the following primers: 5′-ATTTCTAGAAGCCACTGGTAACCACGC-3′ and 5′-ATTAAGCTTCGACGGGACCTGATCTCC-3′ for the *snoaM* gene from *S. nogalater* and 5′-GATTCTAGAGTCACTGGAGCGAAGGTT-3′ and 5′-GATAAGCTTCGGAACGTTCATTCGTCG-3′ for the corresponding cyclase genes from wild type

S. peucetius and from the *S. peucetius* mutant D2. PCR was carried out with 25 pmol of each oligonucleotide primer, 1 ng of plasmid template, 0.1 mM of each dNTP, 3% DMSO and 0.8 U of DyNAzyme EXT DNA polymerase (Finnzymes, Finland). The template was initially denaturated by heating at 99°C for 8 minutes followed by 30 cycles of amplification, *i.e.*, denaturation at 96°C for 1 minute, annealing at 59°C for *snoaM* and at 65°C for the cyclase genes from *S. peucetius* wild type and D2 strains and extension at 73°C for 1.5 minutes. The reaction was completed with additional extension for 8.5 minutes. The PCR products obtained were cloned in *E. coli* using a TOPO TA Cloning kit (Invitrogen), according to the manufacturer's instructions and verified by sequencing. The DNA used for sequencing was purified by a Silica Spin Disc Plasmid DNA Miniprep kit (Biometra). DNA sequencing was performed using the automatic ABI DNA sequencer (Perkin-Elmer), according to the manufacturer's instructions. Sequence analysis was carried out using the GCG sequence analysis software package (Version 8, Genetics Computer Group, Madison, Wisconsin, USA).

Expression Constructs

The polyketide cyclase homologue from *S. nogalater*, *snoaM*, amplified by PCR was cloned into pIJE486 downstream of *ermE* promoter, and into pMC9 (Kantola, unpublished) to obtain pSYE66 and pMC10aM, respectively. pSYE66 and pMC10aM were introduced into *S. lividans* TK24. Plasmids pSY42[16], pSY21[8], pSY15[17], and pSYE66 isolated from TK24 were further introduced into D2. Similarly, plasmids pSgs4 and pSgs44 containing the polyketide cyclase homologue from *S. galilaeus*[18] and a plasmid pDpsY containing *S. peucetius* wild type cyclase amplified by PCR were introduced into D2. In addition, pMC9 and pMC10aM were introduced into *S. coelicolor* CH999. The plasmid constructs used are listed in Table 1.

Cultivations

Liquid cultivations for studying anthracycline production were performed in 250-ml Erlenmeyer flasks containing 60 ml of E1 medium consisting of glucose 2%, starch 2%, Pharmamedia 0.5% (Traders protein), yeast extract 0.25%, $CaCO_3$ 0.3%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ 0.1% and K_2HPO_4 0.1% in 1 liter of tap water (pH 7.5)[19]. Fermentation was carried out for 6 to 7 days in 10 liters of E1 medium. Mutagenization and preparation of plasmid DNA were carried out in TSB medium. The plasmid-carrying strains were grown in the presence of 5 μg/ml thiostrepton in liquid medium and 50 μg/ml in solid medium (ISP4 or R2YE). For *E. coli* and *Streptomyces* strains, the general culture conditions were as described in SAMBROOK *et al.*[15] and HOPWOOD *et al.*[12].

Detection of Metabolites

A 250-μl sample of E1 culture was adjusted to pH 7.0 by 250 μl of 1 M potassium phosphate buffer, and subsequently extracted with 250 μl of MeOH and 500 μl of $CHCl_3$. The solvent layer was concentrated, and 1~2 μl was spotted on a precoated Kieselgel 60 F_{254} glass plate (E-Merck & Co.), and developed with $CHCl_3$-MeOH-AcOH, 20:5:1 (in volume) or toluene-EtOAc-MeOH-HCOOH, 50:50:15:3 (in volume). The production profile of the D2 mutant and the purity of the fractionated compounds were determined by HPLC on a Hewlett Packard 1100 series chromatograph equipped with a LiCHroCART (55×4 mm) RP-18ec column and a diode array detector. The mobile phase consisted of a gradient elution with 0.1% formic acid and MeCN. The flow rate was 1 ml/minute. A mobile phase used for the separation of anthracycline compounds was MeCN-KH_2PO_4 buffer (60 mM, pH 3.0 adjusted with citric acid). The compounds were separated with a gradient from 65% to 25% KH_2PO_4 buffer (60 mM, pH 3.0). The flow rate was 1 ml/minute, and detection was done at 254 and 480 nm.

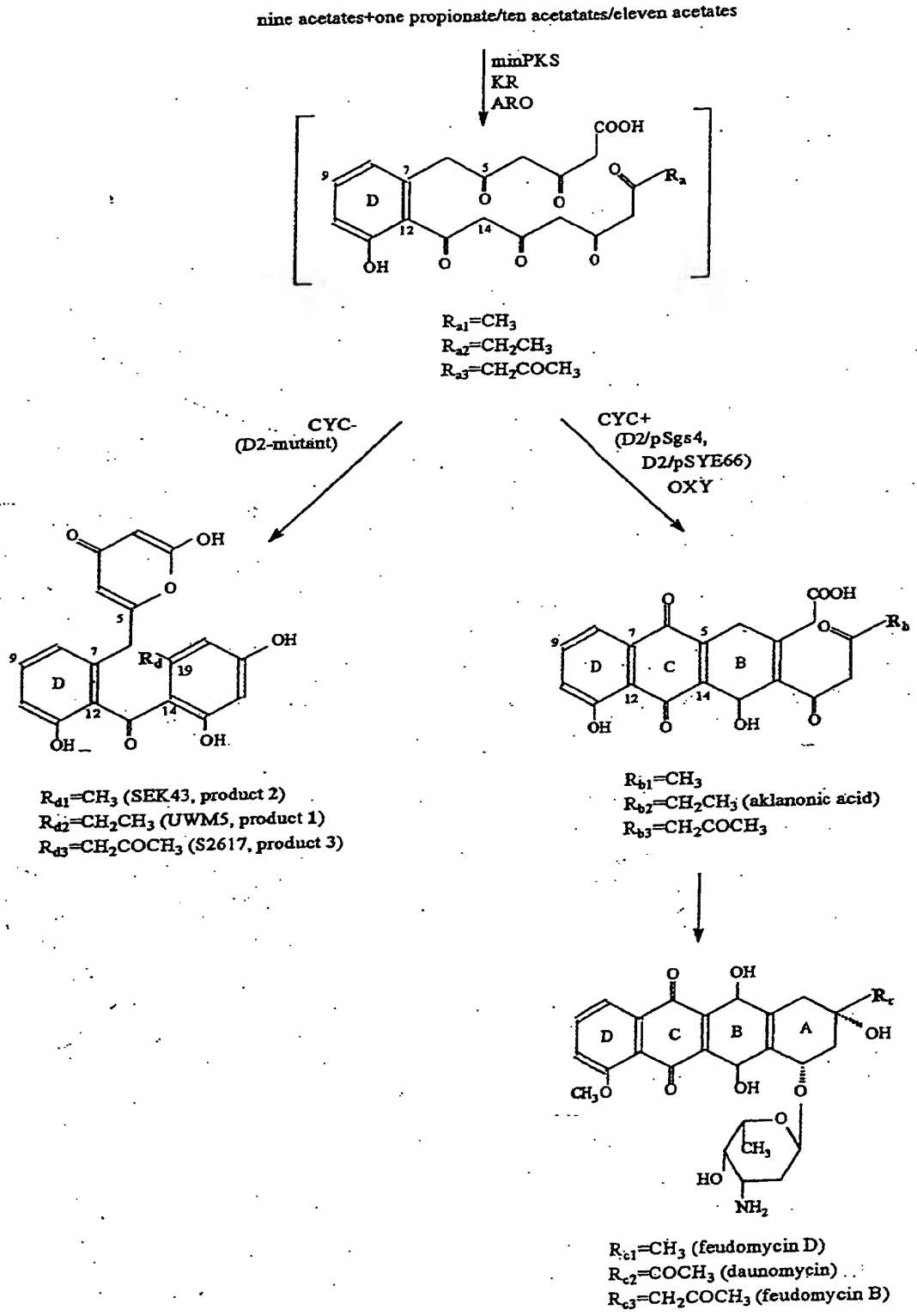
Purification of Metabolites from D2

The fermentation broth (10 liters) was adjusted to pH 3.0 prior to processing. Cells were separated from supernatant with centrifugation, and extracted with 2.5 liters of methanol. Supernatant was treated with 250 g of XAD-7 resin for 1 hour. The products were eluted from the resin with 2 liters of methanol. The combined cell and supernatant extracts were treated with water and subsequently extracted twice with 2 liters of chloroform. The organic layer was evaporated to dryness. HPLC analysis of the residue revealed one major (70% of the integral at 254 nm) and several minor products. The viscous residue was loaded into a (7×10 cm) silica flash column. The column was washed with 1% acetic acid in chloroform, and eluted with a linear methanol gradient up to 30%. Three pooled fractions were further purified in a semi-preparative RP-18 ec column (20×2.5 cm) eluted with a descending gradient of 1% acetic acid and MeCN. Evaporation of MeCN resulted white powdered products, dried under vacuum, yielding 350 mg of product **1**, 22 mg of product **2** and 15 mg of product **3**.

Spectroscopy

NMR spectra were taken on a JEOL JNM-400 spectrometer operating at 400 MHz and 100 MHz for proton and

Fig. 2. Structures of the products obtained from *S. peucetius* D2 and the complementation of the mutant with cyclase containing plasmids which restored the production of daunomycins.



Abbreviations: min PKS=minimal polyketide synthase, KR=polyketide reductase, ARO=aromatase, CYC=second/third ring cyclase, OXY=mono-oxygenase. Only the key intermediates are shown and the arrows are representing multiple steps in biosynthesis. Structure in parenthesis is hypothetical.

carbon respectively, using either a 5-mm normal or an inverse configuration probe. The samples were measured in DMSO-d_6 at 26°C and internally referenced to tetramethylsilane. For HSQC and HMBC measurements, preemptive Bird pulse was employed. EIMS spectra were taken on a VG Analytical Organic mass spectrometry 7070 E. UV spectra were recorded on a Pharmacia biochrom 4060 spectrophotometer in methanol.

Results and Discussion

Characteristics and Products of D2 Mutant

D2 was obtained from the mutagenesis of the wild type *S. peucetius* var. *caesius*. It grows as colorless colonies on ISP4 agar plates, while the wild type has a light orange color. In liquid cultures, D2 did not produce any detectable amounts of anthracyclines, whereas the wild type produces a mixture of baumycins[20,21]. Baumycins are daunomycin-derivatives with additional sugars attached to daunosamine.

The UV spectra of purified products **1**, **2** and **3** (Fig. 2) showed similar chromophores with a substituted aromatic ring. The ^{1}H NMR spectra indicated two aromatic rings and a 2-hydroxy-4-pyrane ring. Furthermore, the spectra showed four hydroxyl groups, confirmed by saturation transfer upon irradiation of water, which resonated between 9.0 and 14.0 ppm. Two of them were sharp and concentration-independent, indicating an intramolecular hydrogen bonding. In the ^{1}H NMR spectra **1**, **2** and **3** differed only in the substitution of one aromatic ring. The ^{13}C NMR spectra gave 21, 20 and 22 carbons for **1**, **2** and **3**, respectively. The carbons were unambiguously assigned using pHSQC and HMBC measurements. The measured values were in good agreement with the known similar structures UWM5 for product **1**[22] and SEK43 for product **2**[13]. The novel compound **3** had a structure similar to SEK43 except for the extra acetate in the side chain at C-19. The assignments for product **3**, designated as S2617, are given in Table 2. Furthermore, the EIMS gave the correct molecular masses and degradation patterns consistent with the structures.

Of the three products D2 strain produces, UWM5 (**1**) was the main compound, while SEK43 (**2**) and S2617 (**3**) were minor ones. Their structures revealed that a ketoreductase (KR) had reduced the carbonyl group at C-9 of the polyketide skeleton. KR is the first enzyme to act on the nascent polyketide chain, and it induces an aldol condensation between C-7 and C-12[23]. In each identified structure the first ring was also correctly aromatized, indicating that the first ring cyclase/aromatase had acted normally. A typical second ring closure for anthracyclines

Table 2. ^{1}H NMR and ^{13}C NMR spectroscopic data of S2617.

Site	^{1}H/ppm, mult., J_{HH}/Hz, area	^{13}C/ppm (multiplicity)
1	-	170 (s)
1-OH	11.55, brs, 1H	-
2	5.16, d, 2.1, 1H	88.2 (d)
3	-	164.2 (s)
4	5.64, d, 2.0, 1H	99.5 (d)
5	-	165.0 (s)
6	4.38, brs, 2H	38.5 (t)
7	-	137.5 (s)
8	6.76, dd, 7.2, 1.3, 1H	128.2 (d)
9	7.19, dd, 8.1, 7.3, 1H	134.7 (d)
10	6.73, dd, 8.1, 1.3, 1H	118.2 (d)
11	-	156.2 (s)
11-OH	9.78, s, 1H	-
12	-	120.0 (s)
13	-	199.6 (s)
14	-	113.4 (s)
15	-	158.2 (s)
15-OH	12.67, s, 1H	-
16	6.08, d, 1.5, 1H	101.3 (d)
17	-	162.0 (s)
17-OH	10.41, brs, 1H	-
18	6.14, d, 1.6, 1H	118.1 (d)
19	-	140.8 (s)
20	4.11, s, 2H	49.5 (t)
21	-	204.0 (s)
22	2.17, s, 3H	30.2 (q)

was expected between C-5 and C-14 but this reaction had not proceeded in a normal way. Instead, the remaining polyketide tails had folded spontaneously, leading to the shunt products (**1**, **2**, **3**) obtained.

The changes in the side chains at C-19 are presumably due to the flexibility of PKS. UWM5, which is a condensation product of nine acetates and a propionate, is a shunt product of daunomycin biosynthesis: SEK43 derived from ten and S2617 from eleven acetates are also shunt products formed from intermediates of *S. peucetius* products; feudomycin D[24] and feudomycin B[25], respectively. The amounts of the D2 products correlated with the amount of the corresponding anthracycline products in the wild type.

Heterologous Complementation of the D2 Mutant

The plasmids, containing biosynthetic genes for nogalamycin[26] and aclacinomycins[27] (see Fig. 1 for structures), derived from *S. nogalater* and *S. galilaeus* respectively, were introduced into D2 by protoplast transformation. Plasmid pSY21 carrying minimal PKS genes for nogalamycin[8] did not complement the D2 mutant. D2/pSY15 also remained non-producing. Although plasmid pSY15 was previously suggested to contain all the genetic information from *S. nogalater* to produce the first three rings of nogalamycin[17]. D2 was further transformed with pSgs4 which carries genes for aclacinomycin biosynthesis, and with pSY42 which contains nogalamycin biosynthetic genes other than pSY21 and pSY15. As a result, D2 accumulates daunomycins as the wild type and shunt products were no longer detected. Interestingly, sequence analysis revealed that both, pSgs4 and pSY42 contained a gene for putative cyclase, *aknW* and *snoaM*, respectively. pSgs4 was further subcloned revealing that pSgs44 expressing *aknW* alone was able to complement D2. Furthermore, D2 was complemented with plasmid pSYE66 containing *snoaM*. The complementation results clearly indicate that D2 is a cyclase deficient mutant.

Influence of Endogenous Enzyme Activities of TK24 on Studying Gene Functions

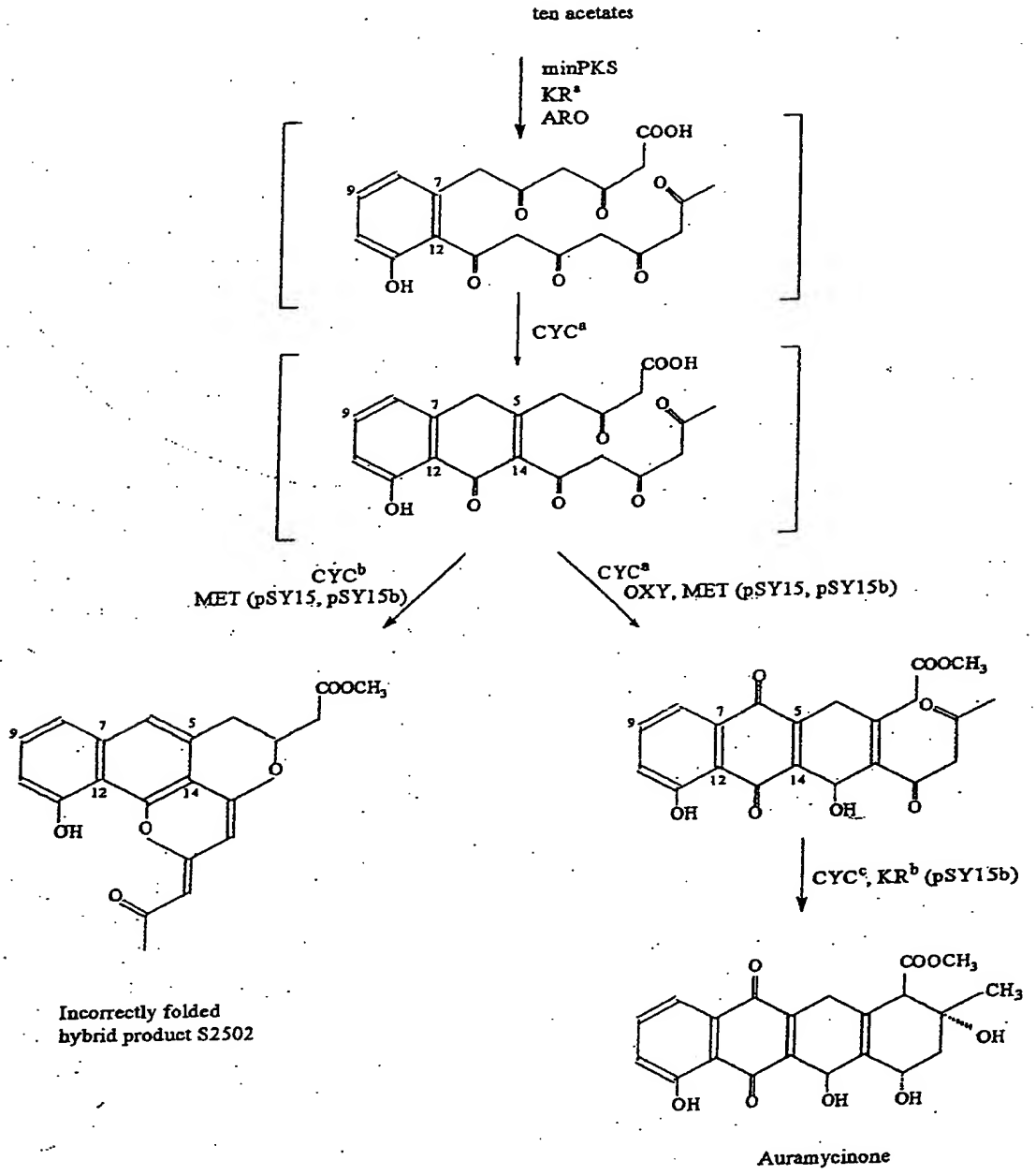
Elucidation of the role of specific enzymes can be problematic due to endogenous activities of the host, which may cause misinterpretation of the results. *S. lividans* TK24 strain, which is commonly used as a host for expression studies, occasionally produces actinorhodin on ISP4 plates, but the production is suppressed in a liquid medium E1. In contrast to results that the second ring cyclase, Act IV[28] acting on actinorhodin biosynthetic pathway in *S. coelicolor* can not act on longer polyketides than octa- and nonaketides in CH999[13], our studies suggest that the corresponding endogenous TK24 cyclase has a role to play in biosynthesis of such polyketide compounds. For example, in our previous studies, *S. lividans* TK24 carrying pSY15 was able to produce aromatic polyketides with correctly closed first three rings although the construct did not contain *snoaM* or a related cyclase gene[10,17]. Furthermore, TK24 carrying pMC9 (Kantola, unpublished) or pSY15b[10] was able to produce minor amounts of auramycinone. Construct pMC9 contains nine anthracyclinone biosynthesis genes derived from three different *Streptomyces* species but no cyclase corresponding to *snoaM* or *aknW*, while pSY15b contains the same genes as pMC9 and an activator, *snorA*. Similarly RAJGARHIA and STROHL[9] were able to produce AA in TK24 transformants and GERLITZ *et al.*[29] in *S. lividans* 1326 carrying genes cloned from *S. peucetius* without genes corresponding to second and third ring cyclases. To get more information whether the cyclizations in TK24 occur spontaneously or by the action of endogenous TK24 enzymes, we expressed pSY15 in a number of unidentified *Streptomyces* strains that do not produce aromatic polyketides in nature. As expected, similar products as in TK24/pSY15 were not obtained (data not shown). If the cyclization was a spontaneous reaction we should have observed related products as were found in TK24/pSY15.

In addition to correctly folded polyketides, TK24/pSY15 and TK24/pSY15b produced incorrectly folded compounds. In these incorrectly folded compounds[17] (Fig. 3) the first two rings are closed correctly but the remaining tails form a tetrahydropyran ring as in actinorhodin, which is an aromatic polyketide product of *S. lividans*. This reaction was most probably catalyzed by a product of the locus corresponding to *actVI*, which acts on the formation of the tetrahydropyran ring found in actinorhodin (Fig. 1) produced by *S. coelicolor*[30]. This further supports the expression of endogenous genes in TK24. It seems that ActIV and ActVI functions are competitive in TK24/pSY15 and TK24/pSY15b after the second ring closure because both correctly and incorrectly folded products were detected (Fig. 3).

Expression of *snoaM* in *S. lividans* TK24 and *S. coelicolor* CH999

To test the activity of *snoaM* on auramycinone production, we cloned it into pMC9 to obtain pMC10aM. As expected, the expression of pMC10aM in TK24 resulted

Fig. 3. Proposed biosynthetic pathways leading to the obtained products in TK24/pSY15[17] and TK24/pSY15b[10].



Abbreviations; min PKS=minimal polyketide synthase, KR[a]=polyketide reductase, ARO=aromatase, CYC[a]=second/third ring cyclase (ActIV), CYC[b]=cyclase involved in the formation of tetrahydropyran ring (ActVI), MET=methyl transferase, OXY=mono-oxygenase, CYC[c]=fourth ring cyclase, KR[b]=aklaviketone reductase. All enzyme activities except those of CYC[a] and CYC[b] are derived from plasmids pSY15 and pSY15b. Only the key intermediates are shown and the arrows are representing multiple steps in biosynthesis. Structures in parenthesis are hypothetical.

in a ten-fold increase in auramycinone production compared with TK24/pMC9. To further clarify the involvement of the TK24 host strain activities pMC9 and pMC10aM were introduced into *S. coelicolor* CH999, which is genetically modified strain to lack the genes needed for actinorhodin biosynthesis and thus should not possess endogenous activities influencing on formation of the metabolites. As expected, only CH999/pMC10aM, carrying all the genes for the aglycone formation, was able to produce auramycinone. CH999/pMC9 remained non-producing. These results confirm that the products obtained from the TK24 strain carrying plasmids without a second and third ring cyclase were produced due to the action of TK24 endogenous enzymes.

Sequence Analyses

Comparison of the deduced amino acid sequence encoded by *snoaM* with database sequences revealed a high degree of similarity to other putative polyketide cyclases found in anthracycline clusters so far. Identities of 73% to AknW from *S. galilaeus*[18], 71% to ORF1 from *S. griseus*[31] and 71% to DpsY from *S. peucetius*[22] were found. Disruption of *dpsY* in *S. peucetius* ATCC29050 has led to production of UWM5[22], the major product of D2 also (product **1**). BAO *et al.*[32] further investigated the role of *dpsY* in the aglycone biosynthesis. They studied constructs with and without *dpsY* transformed in *S. lividans* 1326. Their studies revealed that the products obtained were either aberrantly cyclized shunt products or AA depending on whether a construct contained *dpsY* or not. In addition to cyclases found in anthracycline biosynthetic clusters, SnoaM showed a 65% identity to MtmY[33] implicated in mithramycin biosynthesis. On the contrary, SnoaM did not show any similarity to cyclase ActIV or to those of the other genes involved in actinorhodin biosynthesis. However, this does not exclude the possibility that ActIV has effect on anthracycline biosynthesis.

To further clarify the nature of the mutation in D2 strain a cyclase designated as *dpsY*[22] was amplified with PCR from wild type and D2 strains. Sequence studies revealed that there is one amino acid difference in the sequences changing glycine at position 191 to serine. All cyclases for the second and third ring closure identified from anthracycline gene clusters sequenced so far (SnoaM, AknW[18], ORF1[31] and DpsY[22]), as well as MtmY[33] from the mithramycin cluster have a glycine at position 191. In addition, there are two other differences in deduced amino acid sequences of wild type and D2 compared to the *S. peucetius* ATCC 29050 *dpsY*[22] sequence in the gene bank (accession number AAC38443): histidine at position 38 is replaced by leucine and aspartic acid at position 126 is replaced by glutamic acid. To confirm the results obtained from heterologous complementation studies we expressed wild type *dpsY* gene in D2. As expected D2 mutant was complemented with the wild type cyclase.

Conclusion

The results from *snoaM* expression studies in TK24 and CH999 confirms the necessity of a specific second and third ring cyclase in anthracycline biosynthesis. Furthermore, complementation of D2 with a cyclase gene from two other *Streptomyces* strains and also with the wild type *S. peucetius* cyclase are consistent with the suggested function of this enzyme. The point mutation changing glycine to serine at position 191 in D2 *dpsY* cyclase seems to be crucial to the activity of the enzyme, since all the sequences for the second and third ring cyclases in anthracycline biosynthesis available in the gene bank have a glycine at this position.

It is most likely that the third ring closure proceeds by the action of the same enzyme that acts on the second ring closure, because there are no reports of identified natural intermediates or shunt products from mutants with the first two rings closed correctly. In addition, the results obtained by expressing *snoaM* in TK24 and CH999 suggest that this enzyme is involved in closures of both the second and third rings. Since anthracycline biosynthesis proceeds similarly in different strains and because the sequence similarities of the PKS regions are high, it can be suggested that the identified *S. galilaeus* and *S. nogalater* cyclases have similar functions in their natural context in the biosynthesis of aclacinomycins and nogalamycin, respectively. Finally, though the concomitant action of genes for different antibiotics may cause confusing results as has been discussed in this paper, it provides a powerful tool to generate novel chemical structures for drug discovery.

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